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NEURODEGENERATIVE MECHANISMS IN ALZHEIMER'S DISEASE

AMYLOID AGGREGATION, NEUROINFLAMMATION AND APOPTOSIS

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Dedicated to the memory of my grandmother Ruža Jelić

CHAPTER 1

General Introduction

"Men ought to know that from nothing else but the brain come joys, delights laughter and sports, and sorrows, grief, despondency, and lamentations. And by this in an especial manner we acquire wisdom and knowledge, and see and hear and know what are foul and what are fair, what are bad and what are good, what are sweet and what are unsavoury... And by the same organ we became mad and delirious, and fear and terrors assail us... All these things we endure from the brain when it is not healthy..."

Hippocrates, *On the Sacred Disease* (Fourth century B.C.)

Part of this chapter has been accepted for publication in Behavioural Brain Research. The Basal Forebrain Cholinergic System in Ageing and Dementia. Rescuing Cholinergic Neurons from Neurotoxic Amyloid-β42 with Memantine. Csaba Nyakas, Ivica Granic, Laszlo G. Halmy Pradeep Banerjee Paul G.M. Luiten.

1.1 INTRODUCTION

1.1.1 Neurodegenerative Disorders

Neurodegenerative disorders represent conditions in which cells in the central nervous system are damaged or lost, they have long been viewed among the most enigmatic and intractable problems in medicine. Unlike in other parts of the body, cells in the CNS have only limited regenerative capacity and are therefore extremely vulnerable to damage. Once a cell is lost it can hardly be replaced. Depending on the cell type and area which is affected the loss of cells within the CNS ultimately leads to physiological and behavioral dysfunctions. The cause of neurodegeneration can be of various origins and is often poorly understood.

1.1.2 Protein misfolding in neurodegenerative disease

During the last decade, there is growing evidence that misfolding and aggregation of particular proteins are a possible cause for the development of a subset of neurodegenerative disorders among which Alzheimer's, Parkinson's and Huntington's disease. These insoluble fibrous protein aggregates are generally referred as amyloids and share specific structural traits.

Therefore, Carrell and Lomas proposed a new group of disorders: the conformational diseases or protein conformational disorders (Carrell and Lomas, 1997). Despite the differences in disease progression and clinical symptoms, these disorders share some common features. First of all, they consolidate usually in the fourth or fifth decade of life with characteristic loss of neurons or dysfunction of inter neuronal signaling. The second feature of conformational disorders is the deposition of fibrillar protein, as a result of the misfolding and aggregation of a native protein. It is quite striking that those protein deposits from different diseases have some similar morphological, structural and staining characteristics. Furthermore, these deposits have a high resistance to proteolytic degradation and a fibrillar appearance under electron microscopy (Soto, 2003). The common structural motif of virtually all amyloid fibres are cross-β-sheets in which the peptide strands are arranged perpendicular to the long axis of the 7-10nm fibre (Selkoe, 2003).

1.1.3 Alzheimer's Disease

Alzheimer's disease (AD) and its underlying pathogenic mechanisms will be the main topic of this thesis. Alzheimer's disease is the most common progressive and age related neurodegenerative disorder and was first described in 1906 by the German physician Alois Alzheimer (Alzheimer, 1911). Almost at the same time

Oskar Fischer published detailed histopathological findings associated with AD (Fischer, 1910). Alzheimer and Fisher described two major neuropathological features, which are still considered to confirm the definitive diagnosis of the disease, namely extracellular plaques composed of the fibrillar β-amyloid protein (Aβ), and neurofibrillary tangles (NFT) that consist of paired helical filaments of hyperphosphorylated tau protein. These histopathological lesions are mainly present in the hippocampus and the cerebral cortex, two large forebrain domains that are crucial for memory and other higher cognitive functions. The characteristic pathology eventually leads to the typical clinical symptoms e.g. general cognitive decline, memory impairment and personality changes associated with AD.

The causes of AD are still rather poorly understood, with various etiologies (e.g. genetics, Aβ overproduction, Aβ impaired clearance) leading to amyloid plaques, tangle formation and extensive neuronal death. However, several lines of evidence point to Aβ as the major driving force in the pathogenesis of AD. The amyloid cascade hypothesis proposes that the Aβ peptides form toxic assemblies which initiate several processes leading to neuronal dysfunction and eventually large scale cell death (Haass and Selkoe, 2007).

1.1.4 Epidemiology and genetics of Alzheimer's Disease

The most important risk factor for developing AD is age. After the age of 65 the prevalence of AD in Western societies has been forecasted to double every 5 years (http://www.alz.org). By the age of 80 the number of patients suffering from AD has increased up to 40%. Interestingly, most of the AD cases are sporadic and only 1% are of familial nature. The pathology of sporadic and familial AD are the same, which suggests a common pathogenesis. In order to identify genetic risk factors leading to AD, families with autosomal dominant forms of Alzheimer's dementia have been screened for genes involved in the incidence of AD. Such screening revealed three genes which are involved in the early-onset of AD, namely the genes for presenilin-1 (PS1) on chromosome 14, presenilin-2 (PS2) on chromosome 1, and the gene for the amyloid precursor protein (APP) located on chromosome 21. Carrying a mutation in one of those genes increases the risk of early-onset AD before the age of 60. Most of the mutations in the APP gene accumulate near the cleavage site of the secretases. Mutations in the PS1 and PS2 genes also enhance the amyloidogenic Aβ processing. Individuals with Down syndrome carry 3 copies of the APP gene, which in all cases leads to increased Aβ production and early development of typical AD pathology in their second decade of life (Selkoe, 2005).

Another risk factor that promotes the incidence for the sporadic late-onset AD is the epsilon 4 variant of apolipoprotein E (ApoE ε4). The gene for apolipoprotein E (ApoE) is located on chromosome 19. ApoE is a polymorphic, multifunctional

protein which is expressed by several cell types including liver, kidney, adipose tissue, macrophages and brain cells. ApoE mediates the uptake of cholesterol, triglycerides and other lipids. The exact mechanism how ApoE ε4 is involved the pathogenic process is not fully understood yet. However, accumulating evidence suggest that ApoE ε4 enhances the Aβ aggregation and reduces the Aβ clearance (Mattson, 2004). It has been shown that ApoE is able to bind Aβ *in vitro* and promotes a β-sheet structure in Aβ peptides (Golabek et al., 1996; Wilhelmus et al., 2005).

1.2 MOLECULAR MECHANISMS UNDERLYING ALZHEIMER'S DISEASE

1.2.1 The cholinergic hypothesis of Alzheimer's Disease

The dysfunction and loss of basal forebrain cholinergic neurons and their cortical projections are among the earliest pathological events in the pathogenesis of AD. In addition to the widespread neuronal loss in these brain regions, the evidence pointing to cholinergic impairments come from studies that report a decline in the activity of choline acetyltransferase (ChAT) and acetylcholine esterase (AChE), reduced acetylcholine (ACh) release and a decrease in the levels of nicotinic and muscarinic receptors in the AD brain (Auld et al., 1998). This observation led in the 70's to the postulation of the cholinergic hypothesis for Alzheimer's disease, which is the oldest AD hypothesis (Bartus et al., 1982; Bowen et al., 1976; Davies and Maloney, 1976).

Cholinergic neurotransmission may be a specific target for Aβ, as it has been shown to reduce both choline uptake and ACh release in vitro (Auld et al., 1998; Kar et al., 1998). It has been stated that ACh release and synthesis are depressed and ACh degradation is affected in the presence of Aβ peptides (Auld et al., 2002).

When AChE becomes associated with amyloid fibrils, some of its characteristics, like sensitivity to low pH, change. Therefore, AChE might play an important role in neurotoxicity induced by Aβ. This notion is supported by the observation that Aβ-AChE complexes are more toxic than amyloid fibrils alone (Alvarez et al., 1998).

Although AChE levels are reduced in AD brain, its activity is increased around plaques and in NFT bearing neurons (Talesa, 2001). The increase in activity of this enzyme is likely due to an indirect effect of Aβ, mediated via oxidative stress (Melo et al., 2003), via voltage dependent calcium channels or via nicotinic acetylcholine receptors (nAChRs) of the α7 subtype (Fodero et al., 2004). $A\beta_{42}$ proved to be far more potent then $A\beta_{40}$ in increasing enzymatic activity of AChE. nAChRs are ligand-gated ion channels of which α7-nAChRs is Ca^{2+} permeable. Aβ has been

shown to bind to α7-nAChRs, affecting its nicotinic currents (Pettit et al., 2001) and Erk/MAPK signaling. By binding to α7-nAChRs, $A\beta_{42}$ triggers membrane depolarization due to the Ca^{2+} influx which activates Erk/MAPK (Dineley et al., 2001). However, the relationship between Aβ and the cholinergic system is not unidirectional. There is a considerable amount of evidence that cholinergic dysfunction influences APP metabolism and consequent Aβ production. For example, it has been shown that stimulation of the M1 and M3 muscarinic receptor subtypes increased the release of APP through activation of PLC/protein kinase C (PKC) cascade (Nitsch et al., 1992). BACE expression was also increased by activation of these receptor subtypes (Zuchner et al., 2004). Furthermore, it has also been found that AChE promotes Aβ aggregation, possibly through Aβ-AChE interaction by a hydrophobic environment close to the peripheral anionic binding site of the enzyme, thus promoting fibril formation (Inestrosa et al., 1996).

1.2.2 The tau hypothesis of Alzheimer's Disease

Another hypothesis focuses on a causative role for the microtubule binding tau protein, the main component of the NFTs in AD. NFTs are formed of hyperphosphorylated tau monomers. Several mechanisms have been elucidated which all lead to NFT formation. In vivo studies have demonstrated that kinases such as glycogen synthase kinase 3 (GSK3) and cyclin dependent kinase 5 (Cdk5) can phosphorylate tau (Noble et al., 2003; Spittaels et al., 2000) and both are probably involved in AD. Cdk5 is only active when it is bound to its activator p35. p35 can be cleaved into p25 via calcium dependent proteases (calpains), as a consequence of sustained elevated calcium levels due to oxidative stress, inflammation and excitotoxicity. The Cdk5/p25 complex is more stable then Cdk5/p35 and is thought to be main responsible kinase for tau hyperphosphorylation. Furthermore, the Cdk5 can decrease the activity of phosphatase-1 (PP1) by activating PP1 inhibitors (Shelton and Johnson, 2004). PP1 is mainly involved in the de-phosphorylation of tau. These mechanisms seem to be independent of Aβ, it is however, not impossible that Aβ also could influence these mechanisms indirectly.

These and other studies suggest calpain as a potential target for therapeutic intervention both at early and late stages of AD. Moreover, initial studies using calpain inhibitors in a mouse model of AD showed an encouraging recovery of cognitive function in transgenic mice that were treated with a calpain inhibitor at an early age (Battaglia et al., 2003). However, it remains unclear whether the inhibition of calpains provides protection against neurodegenerative insults, such as excitotoxicity or oligomeric Aβ, as observed in AD.

1.2.3 The amyloid-β cascade hypothesis

The amyloid hypothesis states that the accumulation of Aβ fibrils resulting from an imbalance between production and clearance is the initiating molecular event that triggers the downstream neuropathological conditions in AD (Hardy and Selkoe, 2002). Several observations suggest that tau hyperphosphorylation is a downstream process induced by Aβ (Gotz et al., 2001; Hutton et al., 1998; Lewis et al., 2001; Rapoport et al., 2002). More recent data led to a modified version, the Aβ cascade hypothesis. According to this hypothesis other, less well characterized, soluble, non-fibrillar species of Aβ may be responsible for the damage to brain tissue and subsequent cognitive impairments in AD. This hypothesis has gained increased interest over the past few years and is now more and more supported by experimental findings (Walsh and Selkoe, 2007).

There are several lines of evidence supporting the notion of a causal role for Aβ. AD like neuropathology is invariably seen in Down's syndrome patients, which results from increased APP expression and consequent higher Aβ levels as can be expected by the localization of the APP gene on chromosome 21 (Prasher et al., 1998). Mutations in or near the Aβ region in the APP gene alter the amount or aggregation properties of Aβ and are sufficient to cause early-onset AD (Levy et al., 1990). Furthermore, Aβ peptides are toxic to cortical and hippocampal neurons (Deshpande et al., 2006). Mutations in presenilins, which constitute the catalytic site of β- and γ-secretase increase the $A\beta_{42}/A\beta_{40}$ ratio and cause very early forms of AD (Kumar-Singh et al., 2006). More evidence is provided by experiments from mice transgenic for human APP which reveal an increase in extracellular Aβ, neuropathological and behavioral changes similar to those seen in AD (Ashe, 2005). Finally, injection of Aβ or co-expression of human APP in tau transgenic mice increases tangle formation (Gotz et al., 2001; Lewis et al., 2001).

1.2.4 Amyloid-β precursor protein

The Aβ peptide is part of the larger amyloid precursor protein (APP), which is a type –I transmembrane protein, that belongs to a protein family, that includes APL-1 in C. elegans, APPL in Drosophila and Amyloid Precursor Protein like Protein 1 and 2 (APLP1 and APLP2) in mammals (Zhang and Xu, 2007). These proteins share several conserved domains such as E1 and E2 in the extracellular part and the APP intracellular domain (AICD), however the Aβ domain is only found in APP (Zheng and Koo, 2006). APP consists of a large extracellular N-terminal (aa 28-128) and a short intracellular (cytoplasmic) C-terminal (Reinhard et al., 2005).

The gene encoding for APP is located on the long arm of chromosome 21 in humans. Alternative splicing leads to the production of several APP isoforms,

ranging from 365 to 770 amino acid residues. The major Aβ peptide encoding isoforms are APP695, APP751, APP770, consisting of 695, 751 and 770 amino acids respectively. Whereas APP 751 and APP 770 are mostly found in the brain but also in the rest of the body, APP 695 is predominantly found in neurons. However, it is not known yet why APP 695 shows tissue specificity (Zheng and Koo, 2006).

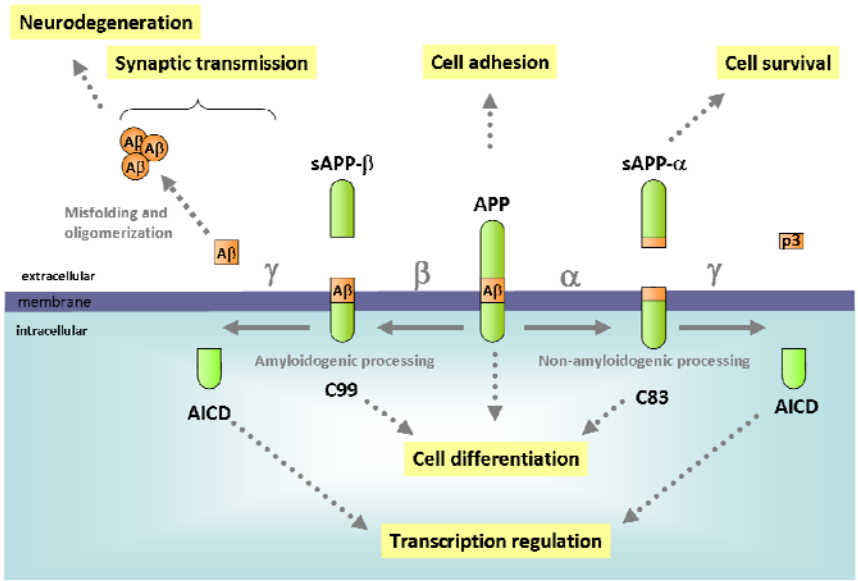

Figure 1.1. *Schematic representation of APP processing and down-stream signals leading to neuronal cell death or survival. Non amyloidogenic APP processing by α- and g-secretase leads to cell survival, whereas amyloidogenic cleavage by β- and γsecretase results in Aβ production and eventually to neurodegeneration.*

1.2.4.1 APP processing and generation of Aβ

Full length APP is synthesized in the endoplasmatic reticulum (ER) and transported through the Golgi to the trans-Golgi- network (TGN), which is the major site of APP residence in neurons at steady state. It is transported to the cell surface in TGN derived vesicles (Zhang and Xu, 2007). APP can be proteolytically processed in two distinct pathways; the amyloidogenic pathway in which Aβ is generated and the non-amyloidogenic pathway (see Figure 1.1) (Zhang and Xu, 2007).

In the non-amyloidogenic pathway, APP is cleaved at the cell surface by α-secretase, a zinc-metalloproteinase, probably consisting of members of the ADAM (a disintegrin and metalloproteinase) family. By cleavage the soluble ectodomain of

APP is released (sAPPα) and a C-terminal stub (CTFα, C-terminal APP fragment α, or C83) is formed. Subsequently, γ-secretase cleaves CTFα which leads to the release of AICD (APP intracellular domain) and the P3 fragment. The γ-secretase is a complex protein consisting of at least four subunits; presenilin (PS), nicastrin, APH-1 and presenilin enhancer-2 (PEN-2). Interestingly, α-secretase cleaves within the Aβ -domain so that no Aβ is formed in this pathway (see Figure 1.1) (Zhang and Xu, 2007).

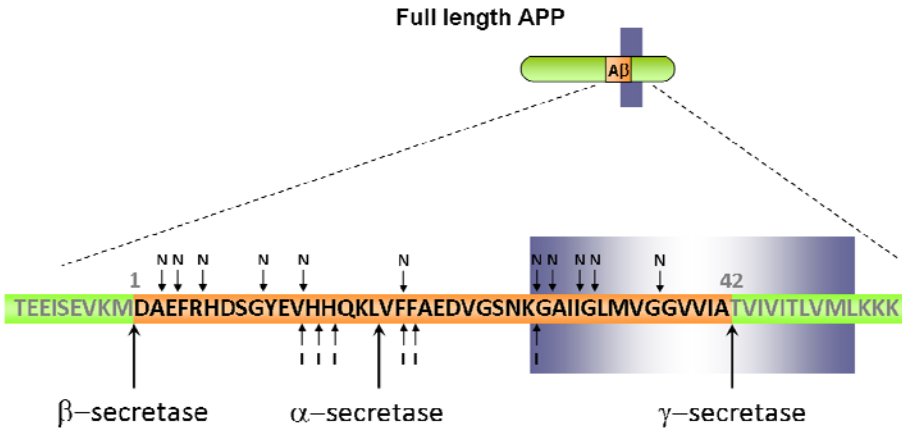

Figure 1.2. *Aβ can be processed by numerous proteases, indicated in the lower part of the panel. The Aβ42 amino acid sequence with cleavage sites for α-, β- and γ-secretase is shown along with: N, Neprilysin (NEP) and I, Insulin degrading enzyme (IDE).*

If APP is not cleaved by α-secretase it can be re-internalized to an endosomal/lysosomal pathway and enter the amyloidogenic pathway where it is cleaved by β-secretase, a transmembrane protease also referred to as BACE1 (β-site of APP cleaving enzyme) (Small and Gandy, 2006). A soluble ectodomain is secreted (sAPPβ) and a C-terminal stub (CTFβ or C99) is generated. Subsequently γ-secretase cleaves CTFβ which leads to the release of AICD and Aβ. The γ-secretase cleaving site is variable, in this way Aβ peptides of different length are generated, ranging from 39 – 43 amino acids (Figure 1.1). The longer Aβ peptides (e.g. $A\beta_{42}$) have the tendency to aggregate more rapidly than shorter Aβ species (e.g. $A\beta_{40}$) and thus form the seed for larger oligomers, fibrils and finally for the macroscopic amyloid plaques (DeMattos et al., 2001; Walsh et al., 2002b; Walsh and Selkoe, 2004). In Alzheimer's diseased brains there is more $A\beta_{42}$ present compared to healthy control brains, whereas $A\beta_{40}$ is far more abundant in healthy brains (Small and Gandy, 2006). Aβ can be degraded by various enzymes such as neprilysin (NEP), insulin degrading enzyme (IDE), endothelin-converting enzyme and plasmin (Figure 1.2) (Pearson and Peers, 2006).

1.2.5 Physiological and pathophysiological effects of Aβ

Like Dr. Jekyll and Mr. Hyde, some apparently innocent peptides have evil alter egos and vice versa. For a long time Aβ was supposed to have only toxic characteristics and to be a waste product of APP cleavage. However, this view is slowly changing as more and more studies find evidence for possible physiological functions of Aβ in synaptic function, memory formation and neuronal survival. In addition, the large amounts of Aβ found in healthy subjects, both in the brain and the rest of the body hints to possible physiological roles (Bishop and Robinson, 2004; Giuffrida et al., 2009; Morley et al., 2009).

1.2.5.1 The role of Aβ in synaptic plasticity and negative feedback in neuronal activity

About 25 years ago Aβ was identified as a major component of the senile plaques in AD (Glenner and Wong, 1984). In human brain it has long been recognized that senile plaque numbers have a weak correlation with severity of cognitive decline (Terry et al., 1991). However, soluble Aβ levels associate well with synaptic loss and severity of dementia (McLean et al., 1999; Wang et al., 1999). Indeed memory impairment and changes in neuron function in APP transgenic mice appear to occur before amyloid deposition (Chapman et al., 1999; Moechars et al., 1999). Animal models of AD show disruption of excitatory synaptic transmission and long-term potentiation (LTP) (Rowan et al., 2003). Walsh and colleagues could show that a low-n oligomeric (dimer, trimer, tetramer) assemblies of naturally secreted human Aβ alters hippocampal synaptic plasticity (Walsh et al., 2002a; Walsh et al., 2005). Also the number of dendritic spines (specialized synaptic sites) was dramatically decreased when neurons were incubated with Aβ oligomers but not with monomers. This decrease in spine density could be reversed when neurons were treated with an anti- Aβ antibody (Shankar et al., 2007). Oligomeric Aβ inhibited neuronal viability 10 fold more than fibrillar Aβ and 40 fold more than monomeric Aβ (Dahlgren et al., 2002).

Glutamate is the most abundant excitatory neurotransmitter in the brain. The glutamatergic receptors α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) and N-methyl D-aspartate (NMDA) receptors (amongst others) are typically found in postsynaptic spines. They have an excitatory effect and can undergo changes in their synaptic strength and efficacy in response to neuronal activity, which is a prerequisite for synaptic plasticity. Long term potentiation (LTP) and long-term depression (LTD) are the most extensively studied forms of synaptic plasticity of the glutamatergic synapse. It is generally considered that synaptic plasticity is the cellular correlate of memory formation (Parameshwaran et al., 2008). LTP is an increase in the communication between two neurons via enhanced synaptic

transmission between excitatory glutamate synapses. LTP is induced by coincident presynaptic glutamate release and postsynaptic depolarization, resulting in postsynaptic calcium influx through NMDA receptors.

The hippocampus is crucial for the acquisition of episodic memory. Impaired acquisition of episodic memory is one of the first symptoms which can be observed in AD patients. It can be correlated with the loss of synapses in the course of the disease (DeKosky and Scheff, 1990; Terry et al., 1991). This and other observations draw the attention to the synaptotoxic effects of Aβ and in particular to deficits in hippocampal synaptic plasticity.

Kamenetz and colleagues could show that Aβ may be a negative feedback regulator of synaptic activity as higher neuronal activity increases the production of Aβ which again leads to a reduced synaptic function and decreases neuronal activity. In this way Aβ might prevent cells from excitotoxicity (Kamenetz et al., 2003). On the other hand, excitatory synaptic transmission is strongly increased in neurons lacking APP mainly due to an increase in synapse numbers (Priller et al., 2006). A possible mechanism could be that Aβ induces the endocytosis of AMPA receptors which leads to decreased surface AMPA receptors and impaired synaptic transmission (Hsieh et al., 2006). Gu and co-workers reported that Aβ reduces CaMKII in synapses which in turn prevents surface delivery of AMPA receptors. This finding links Aβ causally to the loss of synaptic AMPA receptors (Gu et al., 2009).

The degree of aggregation and quantity of Aβ assemblies seem to be critical for the biological processes which are activated by Aβ. Application of micromolar concentrations of $A\beta_{42}$ (a mixture of mature fibrils and protofibrils) attenuated AMPA-evoked neuronal firing whereas application of $A\beta_{40}$ had no effect (Parameshwaran et al., 2007). Other studies using physiological amounts of $A\beta_{42}$ (in the picomolar range) showed an enhancement in hippocampal LTP, whereas nanomolar amounts attenuated LTP (Puzzo et al., 2008). Oligomer secondary structure and degree of assembly correlated directly with fibril nucleation activity. Very recently Ono and colleagues studied specific Aβ oligomers assemblies, which have been stabilized structurally and isolated in pure form (Ono et al., 2009). Those isolated oligomers were tested for neurotoxic activity. Neurotoxic activity which increased disproportionately (order dependence >1) with oligomer order (Figure 1.3) (Ono et al., 2009).

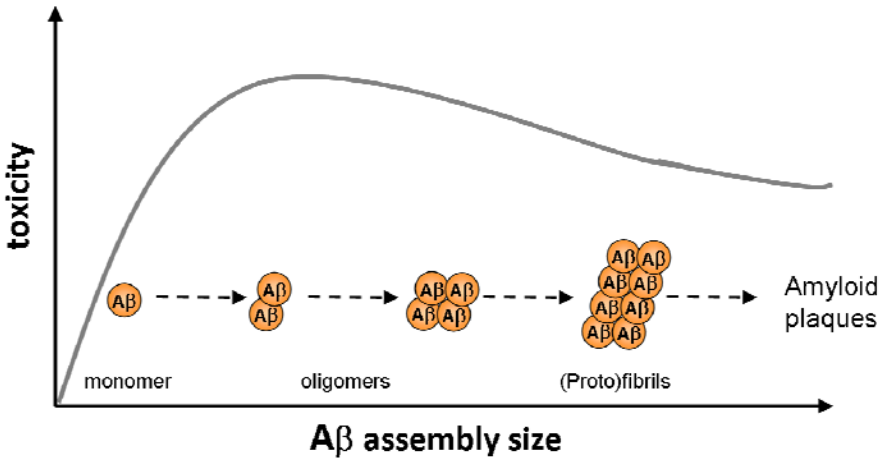

Figure 1.3. *Toxicity and assembly state. Aβ can exist in multiple assembly states – monomers, oligomers, protofibrils and fibrils. In its monomeric state, Aβ does not appear to be neurotoxic. By contrast, oligomeric and protofibrillar species are considered to be most toxic.*

Since Aβ is very hydrophobic and can bind to many cell surface proteins and receptors (Verdier and Penke, 2004) it has been suggested that Aβ directly binds to and modulates AMPA receptors. Indeed, $A\beta_{42}$ iontophoretically applied to neurons from the hippocampal CA1 region attenuated AMPA-evoked neuronal firing whereas NMDA-evoked firing was potentiated (Szegedi et al., 2005). Even a mild abnormal NMDA receptor activation, either via Aβ or by glutamate, could cause neuronal death by initiating cyclic neurotoxic effects in which a shift from α-secretase to β-secretase results in excessive Aβ production and further glutamate accumulation (Harkany et al., 2000; Lesne et al., 2005; Parameshwaran et al., 2008). Aβ oligomers appear to bind to NMDA receptors at the synapse and trigger NMDA receptor internalization and deregulation of NMDA signaling pathways (Lacor et al., 2007; Shankar et al., 2007).

1.2.5.2 Aβ and lipid metabolism

Next to its actions in synaptic plasticity Aβ might also have a crucial role in lipid homeostasis via the regulation of cholesterol and sphingolipid metabolism. The brain contains about 25% of the total body cholesterol and is thus the most cholesterol-rich organ. As cholesterol is essential for development and maintenance of neural plasticity and function, cholesterol homeostasis, including synthesis, removal, storage and transport, must be strictly regulated (Hartmann, 2006).

Cholesterol is very abundant in neurons and glia cells and is essential for Aβ binding to cell membranes and cytotoxicity (Subasinghe et al., 2003). Furthermore, cholesterol can be oxidized by Aβ, leading to 7β-hydroxycholesterol which might be neurotoxic as a pro apoptotic oxycholesterol (Nelson and Alkon, 2005).

1.2.5.3 Oxidative stress

It is currently believed that oxidative stress has a significant role in the pathogenesis associated with Aβ peptides. In AD brain, increased levels of protein carbonyl and nitration of tyrosine residues are found, which is indicative of elevated oxidative modification of proteins (Smith et al., 1996). Protein oxidation is only observed in brain region where $A\beta_{42}$ is present, and not in e.g. the cerebellum, which is largely spared in AD (Hensley et al., 1995). Furthermore, there is also indirect evidence from studies showing that treatment with antioxidants, e.g. vitamin E (tocopherol, TCP) delays the progression of AD (Sano et al., 1997). Aβ peptides have been proposed as a source and a consequence of oxidative stress in an animal model of AD. It has been shown that, an increased load of reactive oxygen species (ROS) is associated with amyloid plaques (McLellan et al., 2003). During Aβ aggregation, H_2O_2 is generated through electron transfer interactions involving bound redox-active metal ions (Curtain et al., 2001; Huang et al., 1999a; Huang et al., 1999b; Opazo et al., 2003). Furthermore, Aβ induces lipid peroxidation and subsequent 4-hydroxynonenal (4-HNE) production, a cytotoxic aldehyde, which can lead to increased vulnerability to apoptosis via JNKs and P38MAPK (Tamagno et al., 2003). Oxidative stress also seems to play a role in Aβ induced neurotrophin mediated neuronal toxicity, which is, however, beyond the scope of this thesis.

1.2.5.4 Insulin signaling

There is evidence that AD is linked to a state of relative brain insulin resistance, also called type III diabetes (de la Monte et al., 2006). In the healthy brain, insulin and insulin-like growth factor 1 (IGF-1) promote glucose utilization and neuronal survival mainly through PI3K/Akt/GSK-3β signaling, which is essential for neuronal metabolism and survival (Bondy and Cheng, 2004). In the AD brain, however, levels of insulin and IGF-1 are deregulated (Moloney et al., 2008). Intracellular expression of Aβ leads to a decrease in phosphorylated Akt levels, an increase in activated GSK-3β, and induction of apoptosis (Magrane et al., 2005). There is recent evidence that intracellular Aβ interrupts insulin signaling by inhibiting 3-phosphoinositide dependent protein kinase (PDK) activity, via interference of binding to its target, Akt (Lee et al., 2009). It was discovered that Akt deactivation is a mediator for oxidative and excitotoxic neuronal death (Luo et

al., 2003). Application of soluble Aβ prevents insulin from binding to its receptor (Xie et al., 2002) and causes loss of surface expression of insulin receptors (Townsend et al., 2007) adding to the above described insulin resistance.

1.2.5.5 Calcium homeostasis

Calcium signaling is of paramount importance for neuronal functioning. Calcium controls membrane excitability, triggers neurotransmitter release, mediates activity-dependent changes in gene expression and modulates neuronal differentiation and transition to apoptosis to mention only a few of its functions (Berridge et al., 1998). Tangle-bearing neurons have increased amounts of calcium and increased levels of calcium-dependent proteases and calcium activated kinases, pointing to a possible role of calcium imbalance in AD pathogenesis (Grynspan et al., 1997). In areas close to amyloid plaques, increased basal intracellular calcium levels are observed in a large proportion of spines and show a strong correlation with the adjacent dendrites. This suggests that local calcium homeostasis has been lost and calcium leaks out of the spine into the dendrite, preventing independent calcium signaling of spines and dendrites (Green and LaFerla, 2008). $A\beta_{42}$ may disrupt intraneuronal calcium levels and regulation by inducing oxidative stress (increased levels of 4-HNE) which impairs membrane calcium pumps and enhances calcium influx through voltage-dependent channels and ionotropic glutamate receptors (Mattson and Chan, 2003). It has also been shown that addition of Aβ leads to abnormal functioning of Na^+/K^+-ATPase (Colom et al., 1998), which in turn could lead to increased levels of intracellular Ca^{2+} via elevated concentrations of intracellular Na^+ which triggers membrane depolarization (Good and Murphy, 1996). In addition, muscarinic acetylcholine receptors (mAChRs) are also targeted by Aβ, leading to Ca^{2+} release from intracellular calcium stores via activation of G-proteins and phospholipase C (PLC) (Kelly et al., 1996).

These excessive and sustained free calcium induces free-radical production by altering mitochondrial oxidative phosphorylation and via the activation of oxygenases, which makes it likely that perturbed calcium homeostasis and free-radicals are components of a self-amplifying cascade (Bezprozvanny and Mattson, 2008). Furthermore, it has been described that in the presence of Aβ peptides there are ion conducting channels formed in the membrane, which could possibly be ascribed to the fact that Aβ oligomers share structural and functional homology with pore-forming bacterial toxins and perforin (Yoshiike et al., 2007). Neurons exposed to phosphatidylserine, which is indicative of apoptotic or energy deprived cells, show enhanced Aβ binding (Lee et al., 2002). Therefore, it is possible that age-related mitochondrial impairments facilitate Aβ-mediated pore formation and calcium influx. In addition, cell-surface receptors coupled to calcium influx are activated and calcium release from the ER is enhanced (Mattson and Chan, 2003). The activation of calpains and caspases and the dysregulation of calcium

homeostasis were shown to be involved in impaired neuronal survival, cell proliferation and differentiation induced by Aβ (Haughey et al., 2002). The main site of calcium dysregulation seems to be the synapse, where Aβ impairs plasma membrane Ca^{2+} ATPase in exposed synaptosomes (Mark et al., 1995). Amyloidogenic processing of APP impairs neuronal calcium homeostasis by decreasing the production sAPPα, a secreted form that activates K^+-channels (Furukawa et al., 1996).

1.2.5.6 Small-conductance calcium-activated potassium channels

Several groups have investigated Aβ effects on membrane ion channels (Davidson et al., 1994; Etcheberrigaray et al., 1994; Etcheberrigaray et al., 1993; Good and Murphy, 1996; Ueda et al., 1997). However, the role of ion channels in Aβ-induced cell death remains controversial and poorly understood. In neurons potassium channels set the resting potential, keep fast action potentials short, terminate periods of intense activity and lower the effectiveness of excitatory inputs. The modulation of neuronal excitability is mediated via calcium dependent potassium channels. These channels allow an efflux of K^+ ions through the plasma membrane, inhibiting subsequent electrical activity (Colom et al., 1998). Small conductance Ca^{2+} activated K^+ channels (KCa2 or SK channels) are strictly voltage-independent, and are activated by changes in intracellular Ca^{2+} levels. KCa2 channels consist of six transmembrane segments (S1-S6), with the pore located between S5 and S6. To activate the KCa2 channel, Ca^{2+} has to bind to calmodulin (CaM), which is associated with the pore-forming channel subunits and serves as the Ca^{2+} sensor. KCa2 channels at spines are activated by Ca^{2+} entry through NMDARs. Low concentrations of Ca^{2+} (300-700nM) are sufficient to open the KCa2 channels, which results in the afterhyperpolarization (AHP) (Alger and Nicoll, 1980; Stocker, 2004). In this way the activation of the channel also counteracts further increases in intracellular calcium (Stocker, 2004). After induction of NMDAR dependent long-term potentiation (LTP), cAMP-dependent protein-kinase (PKA) phosphorylates KCa2.2, resulting in endocytosis and therefore removal of KCa channels (Lin et al., 2008).

There are different subtypes of KCa2 channels (KCa2.1 KCa2.2 KCa2.3) which are predominantly expressed in the nervous system (Kohler et al., 1996; Stocker, 2004; Stocker and Pedarzani, 2000) and show partially overlapping but distinct expression patterns. KCa2.1 and KCa2.2 are the predominant subtypes in cortical regions whereas KCa2.3 is more abundant in subcortical and midbrain regions (Sailer et al., 2002).

1.2.5.8 Excitotoxicity and apoptosis in Alzheimer's Disease

Programmed cell death (apoptosis) is an important biological process during development and under pathological conditions. The most widely studied type of programmed cell death in the nervous system is apoptosis, a process that is regulated by specific cystein proteases called caspases (Yuan and Yankner, 2000). Several different triggers of neuronal apoptosis have been documented in AD, many of them are related to Aβ-mediated processes including oxidative stress, overactivation of glutamate receptors, trophic factor insufficiency, DNA damage and accumulation of damaged proteins (Mattson, 2000; Yuan and Yankner, 2000).

Nevertheless, the over activation (excitotoxicity) is caused by an overstimulation of N-methyl-D-aspartate (NMDA) receptors is widely seen as a common principle in several neurodegenerative disorders including AD (Harkany et al., 2000; Lipton and Rosenberg, 1994; Mattson, 2004). Aβ oligomers have been reported to mediate their pathological effects in vitro via the NMDA receptor (DeFelice and Goswami, 2007; Kelly and Ferreira, 2006; Shankar et al., 2007). Overstimulation of the NMDA receptor leads to excessive entry of calcium into the cell, which activates proteases that are involved in cell death signaling (Chan et al., 1999; Chan and Mattson, 1999; Gingrich et al., 2000). Calpain is such a protease, which can modulate the conformation, localization, and activity of various proteins. The activation of calpain has been linked to a wide range of pathological conditions including Stroke, type 2 diabetes mellitus (T2DM) and AD (Blomgren et al., 2001; Horikawa et al., 2000; Tsuji et al., 1998). As described earlier Aβ exposure can lead to the activation (via elevated Ca^{2+} levels) of calpain, which is due to elevated levels in intracellular calcium homeostasis, which in turn leads to DNA fragmentation by cleavage of poly-ADP-ribose polymerase, a DNA repair enzyme (Boland and Campbell, 2003). For this reason, calpains have been discussed extensively as target for therapeutic interventions in a number of neurodegenerative diseases that are associated with neuronal loss (Goll et al., 2003; Huang and Wang, 2001; Zatz and Starling, 2005).

1.2.5.9 Neuroinflammation

All neurodegenerative diseases are accompanied by activated inflammatory and neuroinflammatory systems. The notion that inflammatory processes are involved in the pathogenesis of AD is also strongly supported by epidemiological studies, indicating that chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD (Stewart et al., 1997). The neuroinflammatory process involves astrocytes, microglia, the complement system and to a lesser extent neurons (Akiyama et al., 2000a). Senile plaques are known to be associated with activated microglia and reactive astrocytes. Microglia interaction with these amyloid deposits triggers the phenotypic activation and as a consequence a number of pro-inflammatory immune receptors and cell surface proteins are overexpressed, such as the leukocyte antigen CD45, complement receptors such as CR3, CR4 and LFA-1,

MHC II surface antigens and the immunoglobulin receptors Fcγ RI, RII and RIII. Moreover, the acute phase proteins amyloid P and C-reactive protein and the protease inhibitors α1-antichymotrypsin and α1-antitrypsin are elevated (Tuppo and Arias, 2005). There is also a large body of evidence reporting that fibrillar Aβ peptides induce the synthesis and release from microglia of pro-inflammatory cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) and the chemokines macrophage inflammatory protein-1 and IL-8 (Yates et al., 2000).

TNF-α is one of the most prominent pro-inflammatory cytokines that is significantly increased in AD. It plays a central role in initiating and regulating the cytokine cascade during inflammatory responses (Akiyama et al., 2000b; Fillit et al., 1991). For example, TNF-α increases the expression of adhesion molecules on the vascular endothelium, which allows leukocytes and immune cells to infiltrate areas of tissue damage and infection (Perry et al., 2001). TNF-α exerts its biological functions via two distinct receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). The 55 kDa TNFR1 (p55/60) is a membrane-receptor and is expressed in most tissues where it can be stimulated by both the membrane-bound and the soluble form of TNF-α. The functions of TNFR1 range from inducing apoptosis and differentiation to NF-κB-mediated cell survival (Hu, 2003). Similar to TNFR1, also the 75 kDa TNFR2 (p75/80) is a membrane-receptor, but because of its low affinity to soluble TNF-α it can be fully activated only by membrane-bound TNF-α. The functions of TNFR2 are as complex as those of TNFR1 and are still not fully clear in all its detail. It is known that the action of the TNF receptors is strongly dependent on the cell type. For instance, TNFR2 is able to amplify apoptotic signals from TNFR1 in cancer cell lines (Wajant, 2003) but has also been reported to mediate protection of nerve cells, as shown in a model for glutamate-induced excitotoxicity (Marchetti et al., 2004b). TNFR2 exerts its protective properties when pre-stimulated with TNF-α, which suggests a neuroprotective role in the CNS (Dolga et al., 2008; Marchetti et al., 2004b). In AD patients, TNFR1 levels are increased (Fillit et al., 1991), whereas TNFR2 (Taoufik et al., 2007) levels are decreased. Recently, it was demonstrated that overexpression of TNFR1 promotes Aβ-induced neuronal death in an APP overexpressing mouse model for AD (Li et al., 2004). In contrast, such APP overexpressing mice lacking TNFR1 have a decreased amyloid plaque burden, lower expression of β-secretase (BACE), and improved learning abilities compared to controls (He et al., 2007). Interestingly, the stimulation of both TNF receptors can lead to the activation of NF-κB, which has binding sites in the promoter regions of both the APP and the BACE gene (Grilli et al., 1996). Mutations in the NF-κB promotor region of BACE lead to a significant decrease in promotor activity of TNF-α activated glia cells or Aβ exposed neurons, which indicates an activating role of NF-κB in BACE expression (Bourne et al., 2007). In this way, NF-κB activation can lead to increased APP expression and enhanced amyloidogenic APP processing. Elevated APP and BACE expression will ultimately lead to increase in Aβ production, which can in turn activate glia cells and enhance neuroinflammatory processes.

1.3 THERAPEUTIC STRATEGIES FOR ALZHEIMER'S DISEASE TREATMENT

Alzheimer's disease has a tremendous negative economic impact since the treatment of AD costs more per patient than management of any other major age-associate brain disease (Golde, 2003) which is one of the factors that explains the great effort which has been made to find ways for a successful treatment strategies. However, the causes of AD are still rather poorly understood, with various etiologies leading to the hallmarks of plaque and tangle pathology, associated neuroinflammation and extensive neuronal death. Therefore it is not surprising that the most advanced strategies for treating this devastating disease have been focused on upstream pathogenic events and preventing Aβ or tau aggregation and thereby protect neurons from death. Currently available therapies however are aimed at rather late processes of the neuropathogenic cascades and for probably that reason only exert limited beneficial outcomes.

1.3.1 Acetyl cholinesterase inhibitors

The reduction and degeneration of cholinergic fibres during aging is a well known phenomenon. The loss of cholinergic transmission is directly associated to memory deficits and cognitive decline (Winkler et al., 1998). The enzyme choline acetyltransferase (ChAT) is the key enzyme for the synthesis of the neurotransmitter acetylcholine. In patients with AD, the ChAT-positive nerve cells are decreased by a factor 4 compared to age-matched, non-demented patients (Pietrzik and Behl, 2005). Based on these findings, in the 70's the cholinergic hypothesis of AD was postulated. This cholinergic hypothesis for Alzheimer's disease, which is the oldest AD hypothesis, stood at the basis for the use of acetyl cholinesterase (AChE) inhibitors, which were the first used pharmacological drugs for the treatment of AD.

At present only five drugs are approved by the U.S. food and drug administration for treating the cognitive symptoms of AD. Four of those drugs (galantamine, rivastagmine, donepezil and tacrine) belong to the class of cholinesterase inhibitors. AChE inhibitors block the disassembly of the neurotransmitter acetylcholine (ACh) by acetyl cholinesterase (AChE). The resulting effect of using AChE inhibitors is an elevated level of ACh in the synapse and thereby enhancing the activity of cholinergic neurotransmission in the AD affected brain regions. Clinical studies showed that the use of AChE inhibitors in patients with mild to moderate AD leads to a modest improvement in learning and memory performance. It should be realized that the usage of AChE inhibitors is only weakening the symptoms and it does not stop any processes leading to AD and its neuropathology. Therefore, the usage of AChE inhibitors is reasonable in combination with other drugs, which intervene in the pathological process of AD.

1.3.2 RAGE inhibitors

Binding of Aβ to the receptor for advanced glycation endproducts (RAGE) is known to contribute to pro-inflammatory processes and neurotoxicity (Deane et al., 2007). Small molecules, which inhibit binding of Aβ to RAGE, like PF-0494700 (formerly TTP488) are able to reduce Aβ toxicity and amyloid aggregation. Currently these compounds are being investigated in a phase II clinical study (Chen et al., 2007).

1.3.3 Modulation of APP processing

The APP processing is a crucial step in the generation of Aβ. β- and γ-Secretase are the enzymes, which are involved in the amyloidogenic pathway. Therefore, they are attractive targets for AD therapy. By inhibiting these enzymes, the generation of Aβ should be reduced, which might in turn slow down the amyloid deposition in the brain.

In case of β-secretase, great efforts have been made by pharmaceutical industries in order to develop potent small molecule inhibitors that can fit the large active site of this aspartyl protease and still penetrate the blood brain barrier (BBB) (Selkoe and Schenk, 2003). For γ-secretase effective inhibitors are already available, but there are concerns about their testing in humans (Hardy and Selkoe, 2002). Both enzymes, β- and γ- secretase may have other substrates which could be essential for the normal functioning of the cell. For example, it has been shown that γ-secretase is involved in the processing of Notch, which has several important and diverse functions *in vivo* (Esler and Wolfe, 2001; Mattson, 2004). Therefore, inhibition of the secretases may have profound toxicological effects and in some cases further developments were abandoned.

1.3.4 Non-steroidal anti-inflammatory drugs

In the 1980s, it was observed that drug treated patients with rheumatoid arthritis had a lower incidence of dementia. Since then scientists have hypothesized that non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and aspirin may delay the onset and risk of Alzheimer's disease (AD) by decreasing the inflammatory changes that occur in the brain (Van Dam et al., 2008). However, the beneficial effects of NSAIDs against AD are not attributed to reduced inflammation but are rather caused by modulating the cleavage of the amyloid precursor protein. In a study to investigate the effect of NSAIDs on the interaction between γ-secretase and APP in cell culture, Hyman and colleagues (Lleo et al., 2004) were able to show that NSAID treatment results in a reduced production of Aβ1-42, whereas the total amount of Aβ did not change. It was suggested that NSAIDs interact with the γ-

secretase and changes the balance of APP cleavage, which results in a reduced production of the toxic Aβ1-42 species by shifting the cleavage towards the shorter non-toxic peptides such as Aβ1-38 (Lleo et al., 2004). A clinical trial with NSAIDs however was aborted in 2005 because of suspicion for causing cardiovascular problems (Gandy, 2005). Another NSAID, tarenflurbil showed in a preclinical study that it modulates gamma secretase cleavage and lowered $A\beta_{42}$ levels in vivo (Eriksen et al., 2003). The drug entered a clinical phase II study where it proved to be safe and well tolerated, however it did not show any benefits on cognition or function in mild to moderate AD patients (Wilcock et al., 2008). A large 18 month phase III clinical trial with tarenflurbil failed to show benefits of the treatment and was discontinued.

1.3.5 Statins

The majority of the epidemiological evidence points to beneficial effect of cholesterol lowering drugs (statins) on AD, although more recent investigations have not always been able to replicate this observation. It is therefore suggested that statins do not prevent AD entirely, but can slow its progression (Hoglund and Blennow, 2007; Wolozin et al., 2006).

HMGR (3-hydroxy-3-methylglutaryl-CoA reductase) is the rate-limiting enzyme in the biosynthesis of cholesterol and catalyzes the conversion of HMG-CoA to mevalonic acid. Drugs like statins completely block this enzyme resulting in lower cholesterol levels (Buhaescu and Izzedine, 2007; van der Most et al., 2009). The mechanism of how statins influence the development of AD is not yet fully understood. However, there are several possibilities how statins might alter AD pathogenesis and exert neuroprotection (van der Most et al., 2009). Statins can reduce cholesterol, which is an important regulator of membrane fluidity. Reduced cholesterol levels could render APP processing by altering the secretase activity, since the cleavage of APP takes place within the membrane. After statin application to primary hippocampal neurons and mixed cortical neurons in culture, Aβ levels were reduced indicating that, when cholesterol levels are lowered, there is a reduced production of Aβ, which is a reversible process (Fassbender et al., 2001). Conversely, a cholesterol-enriched diet increases cerebral Aβ levels in APP-transgenic mice. There is also evidence that cholesterol enhances γ-secretase mediated Aβ production, thus causing a shift from the non-amyloidogenic to the amyloidogenic pathway (Grimm et al., 2007). Furthermore, cholesterol could directly influence the deposition of Aβ or indirectly by altering ApoE levels (Golde, 2003; Mattson, 2004).

Another mechanism of statins was shown by Dolga and colleagues, where pre-treatment of cultured cortical neurons led to protection against excitotoxic damage

(Dolga et al., 2008). Lovastatin was found to increase selectively the expression of TNFReceptor 2. TNF receptor levels, rather than TNF itself, were found to be increased in ischemic tissue and TNFReceptor 2 was found to activate a neuroprotective signaling cascade including PKB/Akt and NF-kB (Fontaine et al., 2002; Marchetti et al., 2004a).

Taken together, the overall benefits observed with statins can not be fully attributed to changes in lipid levels alone, suggesting pleiotropic effects beyond cholesterol lowering, such as improving endothelial function, decreasing oxidative stress and inflammation (James K. Liao, and Ulrich Laufs 2005). Furthermore, it remains elusive whether statin treatment provides protection against neurodegenerative conditions.

1.3.6 Vaccination and immune therapy

Another promising strategy to reduce the plaque load in Alzheimer's disease is immunotherapy. The idea behind this strategy is to inject pre-aggregated synthetic Aβ-peptides into AD patients to elicit an immune response against the toxic amyloid-fibrils and to promote the clearance of amyloid plaques. This approach was validated successfully in an animal model for AD, were the number of amyloid plaques was reduced. The Aβ-vaccination has reached clinical phase II were AD-affected patients received immunotherapy. Sixty-six % of the patients who participated in this randomized trial responded to the Aβ immunization by generating antibodies that recognized the pathological Aβ species but not soluble Aβ or APP (Hock et al., 2002). Unfortunately, the clinical trial had to be aborted because 6 % of the treated patients developed severe inflammatory side effects, e.g. meningo-encephalitis.

1.3.7 N-methyl-D-aspartate receptor antagonists

Accumulating evidence suggests that Aβ-induced neuronal death is caused by N-methyl-D-aspartate (NMDA) receptor-mediated glutamate excitotoxicity (Harkany et al., 2000; Hynd et al., 2004). The central role of the glutamate-induced excitotoxicity in many diseases (e.g. stroke, cerebral-ischemia) was the impulse for the pharmaceutical industry to develop NMDA-receptor antagonists which are capable to block glutamate-induced cell death. Many clinical trials with NMDA receptor antagonists (e.g. MK-801) have failed, because of undesired side effects such as hallucinations, agitation, increased blood pressure and anaesthesia. These side effects occurred because the high binding affinity towards the NMDA receptors interfered with the normal receptor functions (Sonkusare et al., 2005).

More recently the low affinity, uncompetitive NMDA receptor antagonist memantine (1-amino-3,5-dimethyladamantane) has gained increasing interest. Clinical trials have shown that memantine treatment leads to functional improvement in AD patients with severe dementia and it is well-tolerated (Hynd et al., 2004). Based on the successful clinical trials the use of memantine in the modulation of glutamatergic function may therefore represent a novel approach for the treatment of AD.

1.3.8 Aggregation inhibitor peptides

Compounds that block Aβ aggregation and fibril formation provide a further potential therapeutic approach against AD. This can be achieved by short synthetic peptides (also referred as β-sheet breaker peptides or Anti Amyloid Peptides) which, are designed to bind to specific Aβ -regions thereby neutralizing or interfering with the negative properties of oligomeric Aβ species. Pioneering work for this approach was done by Hilbich, Esler and Tjernberg. They identified the amino acid sequence in the amyloid-β peptide which is thought to be responsible for aggregation by replacing amino acids in the hydrophobic core of Aβ (Esler et al., 1996; Hilbich et al., 1992; Tjernberg et al., 1996). Based on this KLVFF (which represents the hydrophobic trans-membrane region of the Aβ peptide) and other sequences, which are known to be involved in aggregation, several aggregation inhibiting peptides were designed. The initially synthesized compounds were peptides of 11 to 5 amino acids targeting the center region of the Aβ peptide and evolved to compounds like LPFFD (iAβ5) and/or LPYFDa. These pentapeptides are partially homologous to this hydrophobic center region and bind with a relatively high affinity to Aβ (Hetenyi et al., 2002a; Permanne et al., 2002; van Groen et al., 2009) by similar intermolecular interactions, leading to a competitive replacement of Aβ molecules. A major drawback with peptide drugs for neurological disorders is their rapid degradation in vivo by proteolytic enzymes and their poor blood-brain permeability (Adessi and Soto, 2002). These issues were overcome by chemical modifications, like C-terminal amidation and N-terminal carboxylation, which resulted in increased half life *in vivo* and rapid brain up-take (Permanne et al., 2002).

1.4 OUTLINE OF THE THESIS

This thesis elaborates therapeutic strategies based on our best current understanding of the pathogenesis of AD. Thereby we try to either slow or halt processes associated with AD pathology. The approaches used in this thesis engage different molecular mechanisms of the disease, such as inflammation, excitotoxicity, Aβ aggregation and toxicity.

Epidemiological studies on cholesterol lowering agents suggest that these drugs might be useful in the prevention of AD. Next to its cholesterol lowering properties, lovastatin induces the up-regulation of TNFR2 in vitro and provides neuroprotection against glutamate induced excitotoxicity. Recent studies have demonstrated that the activation of NF-κB via TNFR2 and PKB/Akt signaling provides neuroprotection and might offer a potential drug target for many neurodegenerative disorders.

In **Chapter 2** the neuroprotective actions of lovastatin were studied in an *in vivo* model for neurodegeneration in which the nucleus basalis is lesioned via NMDA injections. The dysfunction and loss of basal forebrain cholinergic neurons and their cortical projections are among the earliest pathological events in the pathogenesis of AD. We evaluated whether pretreatment with lovastatin prevents degeneration of cholinergic fibers and associated cognitive decline. Furthermore, we investigated whether PKB/Akt signaling is involved in the lovastatin mediated neuroprotection.

Chapter 3 is focused on downstream targets of TNF-α induced NF-κB activation and the underlying neuroprotective mechanism. Recently it has been found that NF-κB binds in the promoter region of KCNN2 and regulates transcription KCa2. KCa2 channels are important regulators of neuronal excitability. Therefore, we investigated if TNF-α signaling is able to induce expression of KCa2 channels and whether this contributes to TNF-α induced neuronal survival against excitotoxic stimuli.

Excitotoxicity is thought to be a major mechanism contributing to neurodegeneration during central nervous system ischemia, trauma, AD and other neurological disorders. Such excitotoxic conditions lead to a disturbance of the intracellular ionic homeostasis and excess intracellular Ca^{2+} ions which can activate Ca^{2+}-dependent proteases (calpains) that eventually lead to neuronal cell death. Therefore the inhibition of calpain provides a promising target to stop neurodegenerative processes. In **Chapter 4** and **5** I evaluated calpain inhibition as therapeutic intervention against NMDA or oligomeric Aβ induced lesion of the NBM *in vivo*. Injection of NMDA or oligomeric Aβ to the nucleus basalis caused a strong deterioration of cortical cholinergic projections leading to deficits in learning behavior. It was tested if the cholinergic denervation and the associated behavioral

decline were prevented by the application of the calpain inhibitor A-705253. Moreover, the extent of microglia activation around the injection site was evaluated.

Inflammatory processes are a hallmark of many chronic diseases including AD and DM. Statistical evidence published in the Rotterdam study and other studies linked DM with an increased risk to develop AD, researchers worldwide have searched for the causal mechanisms underlying this relationship. **Chapter 6** provides an overview on the current literature linking AD and DM via inflammatory processes and NF-κB. Because the NF-κB pathway plays a major role in the aetiology in both diseases, it seems reasonable to assume that mechanisms causing disturbances in this pathway are at the core of the relationship.

The deposition of Aβ peptide into plaques is a defining pathological feature of AD and mutations that alter Aβ generation cause AD. Therefore it seems obvious that blocking the aggregation into oligomers and fibrils is a potential strategy to overcome the toxic effects of this peptide. Compounds which are designed to delay and block Aβ aggregation are Aβ derived short synthetic peptides. In Chapter 7 and 8 the potential therapeutic effects of such aggregation inhibiting peptides was examined. These short synthetic peptides are designed to neutralize the devastating actions of oligomeric Aβ peptide. In **Chapter 7** the aggregation inhibitor LPYFDa is tested for its properties to neutralize Aβ oligomer induced toxicity and memory deficits.

Chapter 8 elaborates the design of new improved aggregation inhibiting peptides using a computer model of an oligomer Aβ to study three dimensional structure of the Aβ peptide/aggregation inhibiting peptide complex to gain more insight into the molecular dynamics of the Aβ aggregation process. The resulting peptide from this design was synthesized and evaluated *in vitro* and *in vivo* for Aβ neutralizing properties.

CHAPTER 2

Pretreatment with Lovastatin prevents N-Methyl-D-Aspartate-induced neurodegeneration in the Magnocellular Nucleus Basalis and behavioral dysfunction

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ABSTRACT

Besides a beneficial cardiovascular effect, it was recently suggested that statins can also exert neuroprotective actions. In a previous study we provided in vitro evidence that lovastatin treatment abates excitotoxic cell death in primary cortical neurons. Here, we investigated the neuroprotective effect of lovastatin in an in vivo mouse model. We found that administration of lovastatin (20 mg/kg) significantly protects cholinergic neurons and their cortical projections against N-methyl-D-aspartate (NMDA, 60 nmol) induced cell death in the magnocellular nucleus basalis (MNB), a neuronal cell group that is characteristically affected in Alzheimer's disease (AD). Furthermore, lovastatin-mediated neuroprotection was shown to be dependent on protein kinase B (PKB)/Akt signaling since treatment with the PKB/Akt inhibitor LY294002 blocked the lovastatin-induced neuroprotective effect. The loss of cholinergic neurons after the MNB lesion resulted in memory impairment as tested in a passive avoidance paradigm. This was reverted by pre- lesion lovastatin treatment. From these studies we conclude that treatment with lovastatin may provide protection against neuronal injury in excitotoxic conditions associated with neurodegenerative diseases including AD.

Chapter 2

2.1 INTRODUCTION

Statins or 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors are commonly used as pharmaceutical compounds to lower cholesterol levels. Besides the beneficial cardiovascular effects statins exert pleiotropic actions in the central nervous system and were suggested to have neuroprotective properties. For example, in a model for brain injury statins (simvastatin and atorvastatin) were shown to increase neurogenesis in the hippocampal dentate gyrus and to reduce neuronal loss in the cornu ammonis 3 (Lu et al., 2007). Simvastatin enhanced neuronal survival in axotomized retinal ganglion cells after optic nerve injury via the overexpression of heat shock protein 27 (Kretz et al., 2006).
In a previous study we showed that lovastatin mediates neuroprotective effects against glutamate-induced excitotoxicity in cultured primary cortical neurons (Dolga et al., 2008). The neuroprotective effect of lovastatin against glutamate-induced excitotoxicity was, at least partly, mediated by the activation of tumor necrosis factor-receptor 2 (TNFR2) signaling pathways, which include protein kinase B (PKB)/Akt phosphorylation and NF-κB activation (Dolga et al., 2008; Marchetti et al., 2004).
A well-established model to study excitotoxic brain damage *in vivo* was originally developed in rats (Dunnett et al., 1987; Luiten et al., 1995; Luiten et al., 1987; Stuiver et al., 1996) and consists of the injection of a neurotoxic dose of the glutamate analog NMDA into the magnocellular nucleus basalis (MNB). Subsequently the neuronal damage is quantified. The basal forebrain cholinergic neurons and their projections to the cerebral cortex are susceptible to experimental conditions generally associated with cerebral ischemia and Alzheimer's disease (AD), including exposure to glutamate, NMDA or amyloid beta peptides (Harkany et al., 1999b; Luiten et al., 1995). Therefore, NMDA-induced lesion of the MNB is an effective *in vivo* method to study neurodegenerative mechanisms associated with NMDA receptor overstimulation (Luiten et al., 1995) and to assess the efficacy of potential therapeutic treatments that may interfere with neurodegenerative processes.
Interestingly, damage to cholinergic MNB neurons and to their cortical target areas is associated with a decline in memory function during aging and in AD and is in fact the basis of the acetylcholinesterase inhibition therapy in AD (Bartus et al., 1982). In the rat model NMDA-induced excitotoxicity in the MNB also led to cognitive deficits (Van der Zee et al., 1994). Hitherto the effects of statins on behavior remain rather unexplored. A few studies, however, showed that statins improve the spatial learning and memory deficits after hypoxic-ischemic or traumatic brain injuries (simvastatin and atorvastatin) in rats (Balduini et al., 2003; Lu et al., 2007) and increase learning and attention (lovastatin) in a mouse model of neurofibromatosis type I (Li et al., 2005). Simvastatin treatment reversed learning and memory deficits in a mouse model for AD (Tg2576 mice). However, simvastatin enhanced also learning and memory performance in the non-transgenic mice, independent of the levels of amyloid-beta protein in the brain (Li et al., 2006).
In the current study, we aimed to substantiate our *in vitro* findings by testing the

protective potential of lovastatin against excitotoxic brain damage *in vivo*. Since our previous *in vitro* studies provided evidence for a role of PKB/Akt in the neuroprotective effect of lovastatin, we also investigated whether PKB/Akt activation is essential in the protective mechanism of lovastatin against NMDA-induced excitotoxicity *in vivo*. To extend the effects of lovastatin in our mouse model we tested memory performance of untreated and lovastatin-treated mice with and without NMDA-induced lesion.

2.2 MATERIAL AND METHODS

2.2.1 Animals and treatments

Experiments were carried out on male C57BL/6J mice (12 weeks, 25-30 g, Harlan, Horst, The Netherlands). The animals were individually housed and kept on a standard laboratory diet and tap water *ad libitum* with a 12/12 h dark-light cycle. All procedures were approved by the Ethical Committee for the use of experimental animals of the University of Groningen, The Netherlands (DEC 4681B).

2.2.2 Nucleus basalis lesion

Animals were anesthetized with avertin (tri-bromo-ethanol) and their heads positioned in a Kopf stereotactic frame adapted to mouse brain coordinates (Kopf Instruments model 900, Tujunga, CA, USA). Unilateral lesions were achieved by a slow (0.1 µl/min) injection of 0.4 µl and a total of 60 nmol NMDA (Sigma, St. Louis, MO, USA solved in phosphate buffered saline, PBS pH=7.4) into the MNB (0.6 mm posterior to bregma, 1.8 mm lateral to the sagital suture, 4.6 mm and 4.4 mm ventral to the dura mater (Franklin and Paxinos, 1997) in two steps of 0.2 µl each with an interval of 5 min. Injections were performed with a Hamilton microsyringe (Hamilton, Bonaduz, Switzerland) in an infusion pump (TSE, Bad Homburg, Germany). Control lesions (0.4 µl PBS) were made in the contralateral MNB of each animal in a manner identical to the NMDA infusion procedure. Thereafter, mice were returned to their home cages and allowed to recover.

2.2.3 Neuroprotective action of lovastatin (experiment I)

In the first experiment we investigated the neuroprotective action of lovastatin against neurotoxic damage induced by a unilateral injection of 60 nmol NMDA in the MBN. In pilot experiments 60 nmol NMDA was shown to induce approximately 50% reduction in cholinergic neurons and projection fibers which was similar to the results found in rat studies in our laboratory (Luiten et al., 1987). Experiment I comprised the following five groups (n = 7 per group). (1) In the first

group mice received 20 mg/kg monosodium salt lovastatin (in DMSO and PBS) (Calbiochem, San Diego, CA, USA) i.p. for 5 consecutive days. Afterwards 60 nmol NMDA dissolved in PBS was injected in one hemisphere and PBS in the contralateral hemisphere. The PBS injected site served as an internal control. (2) In the second group, mice were injected with saline i.p. for 5 consecutive days followed by an unilateral 60 nmol NMDA lesion and PBS infusion contralateral. (3) In the third group, mice received lovastatin and NMDA injections as in group 1) but the PKB/Akt inhibitor LY294002 (100 μM, Calbiochem, San Diego, CA, USA) was infused into the MNB together with NMDA (4). In the fourth group, mice were treated as in group 2) but received LY294002 together with NMDA into the MNB. (5) To check whether LY294002 treatment by itself already affects cholinergic neurons, we infused LY294002 without NMDA into the MNB of saline-pretreated mice. All animals were sacrificed 7 days after the surgical procedure (Figure 2.1A).

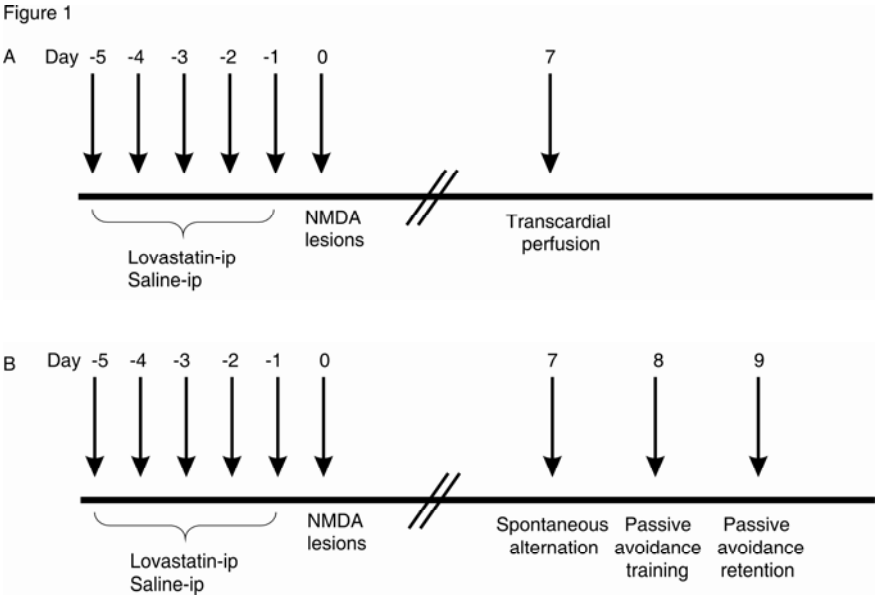

Figure 2.1*. Schematic outline of the experimental setup. Mice were injected for 5 consecutive days with saline or lovastatin (20 mg/kg of body weight) for 5 consecutive days prior to unilateral NMDA lesions (60 nmol) into the magnocellular nucleus basalis. A. Eight days after the last i.p injection mice were transcardially perfused (for histochemical analysis) and their brains fixated with paraformaldehyde. B. Memory performance was assessed using spontaneous alternation and one-way step-through passive avoidance paradigms. These tests were performed eight days after the drug delivery.*

The lovastatin dose given in the above described experiments is based on the results of earlier pharmacokinetic studies (Endres et al., 1998). In this mouse model of focal cerebral ischemia pre-administration of either lovastatin or simvastatin for 3

consecutive days resulted in a slightly reduced cerebral infarct volume when 2 mg/kg statins were administered. However, significantly reduced cerebral infarct volumes were observed after administration of 20 mg/kg statin (Endres et al., 1998).

2.2.4 Behavioral assessment (experiment II)

To study the behavioral consequences of lovastatin treatment and MNB lesions, four groups of animals were formed: (1) mice injected with saline i.p. for 5 consecutive days; (2) mice injected with 20 mg/kg lovastatin i.p. for 5 consecutive days; (3) mice receiving saline for 5 days i.p. followed by bilateral 60 nmol NMDA lesions on the next day; (4) mice receiving lovastatin for 5 days i.p. prior to bilateral 60 nmol NMDA lesions. 8-10 days after the last i.p injection, mice were tested in spontaneous alternation and passive avoidance paradigms (Figure 2.1B).

2.2.4.1 Behavioral testing

2.2.4.1.1 Spontaneous alternation task

Short-term spatial memory performance (working memory) was assessed by recording spontaneous alternation behavior in a Y-maze paradigm. The Y maze consisted of three tubular and transparent Plexiglas arms forming the Y. All three arms were 5 cm in diameter, 27.5 cm long, and at a 120° angle to each other. The experimental room contained visual cues, which served as distal spatial cues.
A naive mouse was placed into the centre of the Y maze and allowed to explore the maze freely during an 8-min session. The series of arm entries was recorded visually. Arm entry was considered to be completed when all four paws of the animal had entered the arm. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The alternation percentage was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two). Since this behavior is not reinforced it is considered "spontaneous". The exploratory activity was assessed by counting the total number of arm entries.

2.2.4.1.2 Passive avoidance paradigm

For this test we used a two-compartment (one-way) step-through device. During the training trial mice were placed in an illuminated compartment, which was separated from the dark compartment by a door. Mice were allowed to explore the light compartment for one minute before the door to the dark compartment was opened. The latency to step into the dark compartment was recorded (pre-shock latency). Upon entry into the dark compartment the door was closed and a mild foot shock (0.3 mA for 2 sec) was delivered through the grid floor. 30 sec after shock

application the mice were returned to their home cages. Memory retention was checked 24 h later. The mice were placed in the illuminated compartment for 20 sec and after opening of the door the latency to step into the dark compartment was recorded (post-shock latency) up to a maximum of 8 min.

2.2.5 Tissue processing and ChAT histochemistry

Fixation of the brains was carried out under deep sodium pentobarbital anesthesia by transcardial perfusion with 200 ml fixative composed of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), which was preceded by a short prerinse (50 ml) with ice-cold physiological salt solution. Brains were post-fixed for 24 h in the same fixative, cryoprotected by 3 days immersion in 30% sucrose in 0.1 M PB at 4°C. Thereafter, 20 µm coronal sections were cut on a Leica cryostat microtome and collected in 0.01 M PBS containing 0.1% sodium azide (Sigma, St. Louis, MO, USA). Free floating sections were pre-incubated overnight at room temperature in 5% normal rabbit serum (NRS, Zymed, San Francisco, CA, USA) with 0.2% Triton-X. The following day, sections were incubated with goat anti choline-acetyltransferase (ChAT) primary antibody (Millipore, Billerica, MA, USA) diluted 1:500 in PBS for 2 - 4 days at 4 °C. Then, sections were exposed to biotinylated rabbit anti-goat IgG (Vector, Brunschwig Chemie, Amsterdam, The Netherlands) diluted 1:500 in PBS followed by an incubation step for 2 h with Vectastain *Elite* ABC kit, according to the manufacturer's protocol (Vector, Brunschwig Chemie, Amsterdam, The Netherlands). For visualization, Sigma Fast 3,3`-diaminobenzidine tablets (Sigma, St. Louis, MO, USA) were used as chromogen with ammonium nickel sulphate (BDH Chemicals Ltd., UK) enhancement. The specificity of immunostaining was confirmed in stainings where incubation with the primary antibody was omitted (data not shown).

2.2.6 Quantitative image analysis

ChAT fiber density was measured in layer V of the posterior somatosensory cortex according to a standardized protocol using an Image Analysis System (Leica Quantimet, Cambridge, UK) with a Leica DFC 350FK camera. The images were analyzed with Leica Qwin Image Analyse software. Surface area density of cortical ChAT positive fibers was measured in parietal cortical sections (0.6 mm posterior to bregma) (Franklin and Paxinos, 1997), the cortical area that receives a strong cholinergic projection from the lesioned MNB region (Gaykema et al., 1990). After background subtraction and grey-scale threshold determination, the surface area of the skeletonized ChAT immunoreactive (ir) fibers ([the area covered by ChAT-ir fibers]/[total sampling area]) was computed in each parietal region using a 470 nm band pass filter. The relative value of ChAT fibers was calculated as the percentage difference between the surface area density of the lesioned hemisphere and the contralateral sham-operated control hemisphere.

2.2.7 Statistics

The statistical test used in this study is ANOVA with LSD or Bonferroni correction using SPSS 14 for windows. A p-value smaller then 0.05 was considered to be statistically significant. Data are presented as mean value ± standard error of the mean (S.E.M.).

2.3 RESULTS

2.3.1 Lovastatin treatment protects against NMDA-induced lesions in mouse magnocellular nucleus basalis

The sensitivity of cholinergic neurons to excitotoxicity can be quantified by the loss of the cholinergic cortical innervation that originates from the MNB (Gaykema et al., 1989) visualized with the cholinergic marker choline-acetyltransferase (ChAT, EC3.2.1.6) (Harkany et al., 2000). Histochemical detection of ChAT-ir projection fibers revealed a dense fiber network in the layer V of the somatosensory cortex with a smooth appearance and a layer specific homogenous staining pattern. The cholinergic fiber density decreased after NMDA infusion proportional to the loss of the number of neurons in MNB (Harkany et al., 2000). Characteristic morphological patterns of neuronal degeneration such as the appearance of shrunken or rounded somatic profiles without emanating dendrites, and fragmented cholinergic fibers ascertained the excitotoxic effect of NMDA-induced lesions (Figure 2.2A and B). Quantification of ChAT positive projection fibers revealed a significant loss of cholinergic innervation in the NMDA-lesion hemisphere compared to the sham-injected hemisphere of 35.33 ± 5.77%. Pre-lesion administration of lovastatin significantly attenuated the NMDA-induced fiber reduction in the parietal cortex (cholinergic fiber loss 11.25 ± 4.2%, $p<0.05$ Univariate ANOVA) (Figure 2.3).

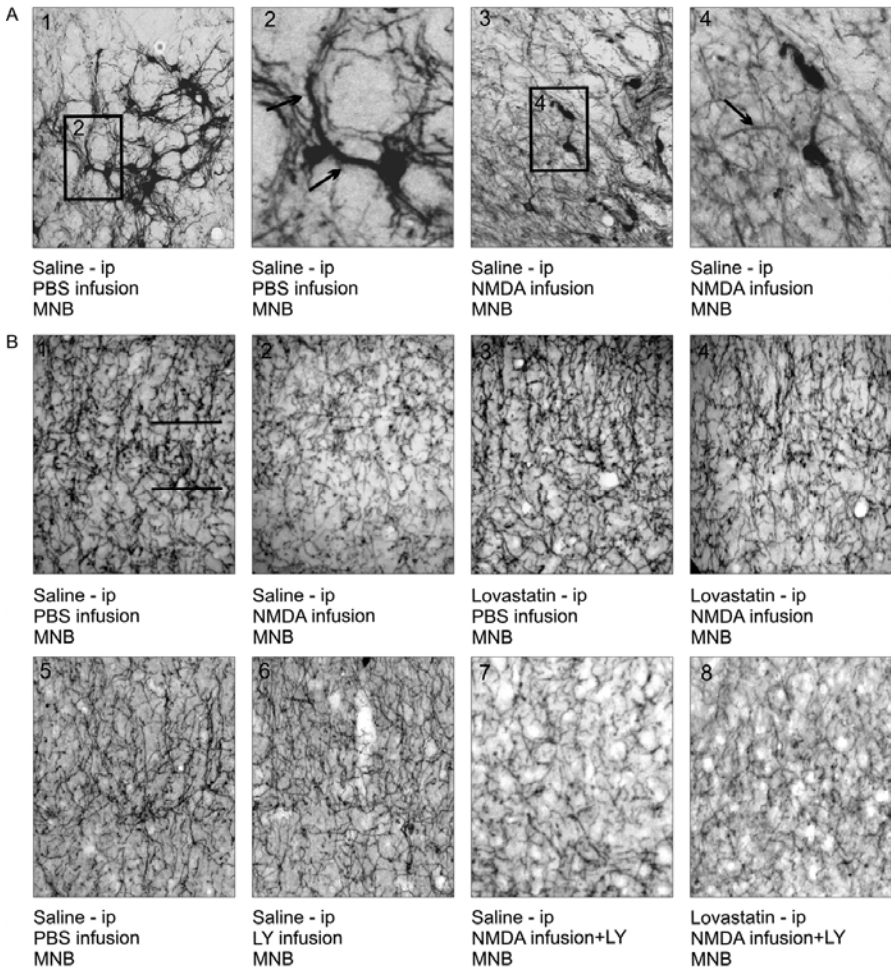

Figure 2.2*. Lovastatin protects cholinergic fibers against NMDA-induced lesions. A. ChAT-ir neuronal perikarya and proximal fiber branches in the MBN following NMDA or PBS infusion. Depicted representative region of intact cholinergic neurons (1, 2) after sham lesions in MNB whereas in (3, 4) it shows the loss of proximal fiber branches emanating from ChAT-ir perikarya. Noteworthy is the loss of cholinergic neurons as a consequence of NMDA toxicity. Arrows show ChAT-ir stained cholinergic processes. B. Distribution of ChAT-positive projection fibers in the posterior somatosensory cortex following NMDA or PBS infusion in the mouse MNB. Depicted representative region of contralateral sham-operated control (1, 3, 5, 7). Noteworthy is the loss of cholinergic projections as a consequence of (2) NMDA infusion. Pre-lesion lovastatin administration (20 mg/kg) significantly attenuated the NMDA-induced damage (4) whereas LY294002 (6, 8) failed to rescue cholinergic projections in lovastatin-treated mice (8). Horizontal bars in (1) represent layer V of the somatosensory cortex where the quantitative measurements were performed.*

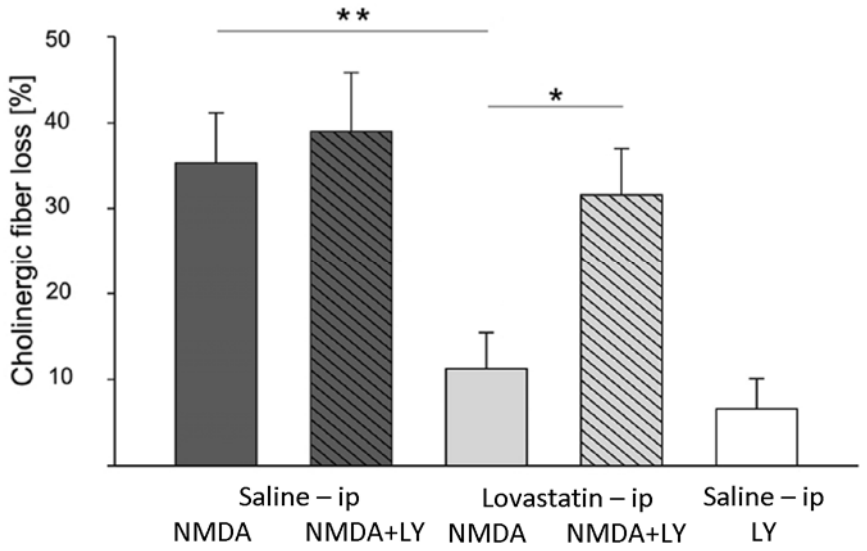

Figure 2.3*. Quantitative measurements of cholinergic fiber densities in layer V of the somatosensory cortex. Whereas NMDA infusion into MNB induced a massive loss of ChAT fiber loss, lovastatin treatment significantly antagonized this NMDA detrimental effect. However, inhibition of PKB/Akt pathway with LY294002 did not rescue cholinergic fiber projections in saline- or lovastatin-treated mice. **P<0.01, *P<0.05 NMDA vs. all other groups examined (Univariate ANOVA), n = 5-7 per group. Data represent means ± S.E.M.*

2.3.2 Lovastatin-mediated neuroprotection is dependent on PKB/Akt activation

Our previous *in vitro* data already revealed an essential role of PKB/Akt pathways in lovastatin-induced protection in primary cortical neurons exposed to glutamate excitotoxicity (Dolga et al., 2008). To further elucidate the lovastatin-mediated neuroprotective pathways *in vivo* we included in our experiments LY294002 as a specific inhibitor of PKB/Akt signalling. The concentration used (100 μM) was based on previous *in vivo* studies, where LY294002 delivered in rat brain by a single injection abolished PKB/Akt phosphorylation (Cheng et al., 2002). This study showed a concentration-dependent effect of LY294002 (100 μM to 800 μM) on PKB/Akt phosphorylation. PKB/Akt phosphorylation was already significantly reduced when 100 μM LY294002 was administrated (Cheng et al., 2002). Lovastatin or saline was administered for 5 consecutive days prior to a combined NMDA and LY294002 infusion into MNB.

Quantitative analysis of the cholinergic fiber loss indicated no effect of the LY294002 treatment alone in the NMDA lesion model (38.67 ± 3.33% for NMDA plus LY294002 vs. 35.33 ± 5.77% for NMDA alone). In the lovastatin treated

animals the ChAT-positive fiber loss in the NMDA plus LY294002 group (31.6 ± 5.41%) showed significantly more fiber loss than the NMDA lesioned cases, which were not treated with LY294002 (11.25 ± 4.2%; $p<0.05$). The lovastatin treated NMDA+LY294002 fiber loss values were not statistically different from the saline-pretreated NMDA nor the saline pretreated NMDA+LY294002 animals. It can thus be concluded that the neuroprotective lovastatin effect is almost completely blocked by LY294002 treatment. Moreover, LY294002 treatment alone did not affect cortical cholinergic fibers (Figure 2.3).

2.3.3 NMDA-induced lesions do not alter spatial short-term memory in a spontaneous alternation task

Four experimental groups of mice (saline i.p.; lovastatin i.p.; saline i.p. and MNB lesion; lovastatin i.p. and MNB lesion) were tested for spontaneous alternation behavior and locomotor activity. The spontaneous alternation test in a Y maze is a task based on the natural tendency of rodents to alternate in their choices of arm visits (Dudchenko, 2004). Spontaneous alternation behavior is generally regarded as a measure of spatial working memory (Dudchenko, 2004; Senechal et al., 2008).
Alternation rates of the mice with MNB lesion showed no significant difference when compared with the non-lesioned animals. Moreover, lovastatin treatment in NMDA-induced lesioned mice did not significantly increase spontaneous alternation rates compared to the saline-treated animals (see Figure 2.4A). Furthermore, we did not find a difference in the number of arm entries between any of the groups, which suggested that the exploratory activity was neither affected by lovastatin treatment nor by MNB lesions (Figure 2.4B).

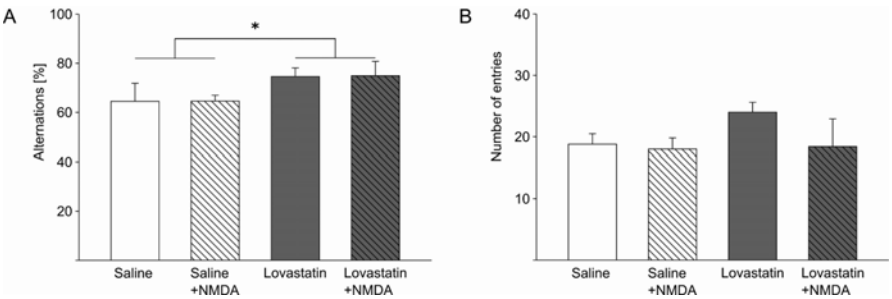

Figure 2.4*. NMDA-induced lesions do not cause changes in working memory A. In the spontaneous alternation task there is no difference in alternation rate between the four groups. Note, however that the pool of lovastatin-treated groups had a significantly higher alternation rate when compared to the pooled two saline-treated groups (p=0.045, two-way ANOVA). B. There was no significant difference in the number of arm entries. *P<0.05, n=6-7 per group. Data are expressed as means ± S.E.M.*

2.3.4 Lovastatin decreased lesion-induced memory deficits

Neuronal projections from MNB directly modulate neocortical information processing. The passive avoidance task is a learning task which depends, in part, on proper neocortical information processing. In rats NMDA-induced lesions in MNB were indeed reported to result in memory impairments in a passive avoidance paradigm (Harkany et al., 1999a; Van der Zee et al., 1994). Therefore, we used this behavioral paradigm to investigate whether preserved cortical cholinergic function in lovastatin-treated mice is able to reverse the cognitive deficits induced by NMDA lesions into MNB.

Altered retention mechanisms and acquisition were investigated at 8 days post-surgery or 8 days after the last intra-peritoneal injection in the passive avoidance paradigm. Pre-shock latencies did not exhibit significant differences among the experimental groups (Figure 2.5A). NMDA infusion into the MNB resulted in a significant impairment of passive avoidance learning. This was indicated by shorter post-shock latencies in the retention trial 24 h after delivery of the foot-shock in the saline-treated NMDA lesion group (4.5 ± 0.75 min) in comparison to the saline-treated group (8 min) (Figure 2.5B). However, lovastatin pretreatment in NMDA lesioned mice significantly increased the post-shock latency time compared to NMDA lesioned animals (6.5 ± 0.8 min vs. 4.5 ± 0.75 min; $p<0.05$ ANOVA with LSD correction) indicative of an improved memory performance in this test condition. Lovastatin treatment alone had no effect on memory performance.

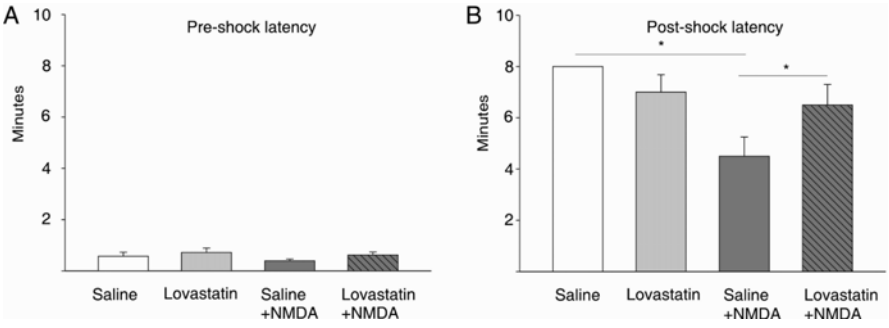

Figure 2.5. *Lovastatin pretreatment decreased the NMDA lesion-induced memory deficits. A. Lovastatin treatment or NMDA infusion in the mouse MNB did not change pre-shock latencies in the passive avoidance test. B. Lovastatin administration by itself did not elicit any significant changes in the post-shock latencies, as compared with the latencies of saline-injected mice. In contrast, NMDA infusion (60 nmol) into the MNB significantly impaired memory retention as shown by the reduced post-shock latencies. However, lovastatin pretreatment (20 mg/kg) attenuated this NMDA-induced memory deficit. *P<0.05 NMDA vs. all other groups examined (ANOVA with LSD correction), n=5-6 per group. Data are expressed as means ± S.E.M.*

2.4 DISCUSSION

In the present study we show, that administration of lovastatin protects cholinergic neurons and their cortical projections against excitotoxic damage *in vivo*. Decline of ChAT positive neurons in the basal forebrain nucleus and their projection fibers in the parietal cortex as a result of excitotoxic stimuli is a well-established technique to test bioactive substances *in vivo* (Dunnett et al., 1987; Luiten et al., 1987). Here, this method was applied for the first time in mice. We could demonstrate a similar outcome of the NMDA lesion as compared with the well-established rat model (Gaykema et al., 1989; Luiten et al., 1995; Stuiver et al., 1996). The advantage of this *in vivo* model is the precisely quantifiable neuronal lesion effect on cholinergic fiber loss. Moreover, because of the strictly unilateral projections of the MNB, the contra lateral sham hemisphere can serve as a control within each individual case. In the current study, five days of lovastatin treatment induced highly significant neuroprotection against NMDA excitotoxicity *in vivo*.

In a recent *in vitro* study, we provided evidence that lovastatin mediates the specific up-regulation of TNFR2 in cultured neurons but not of TNFR1 (Dolga et al., 2008). Interestingly, this TNFR2 up-regulation in combination with TNF signaling led to a strong neuroprotection against glutamate exposure. In several studies we could demonstrate that neuroprotection initiated by activation of TNFR2 is mediated by PKB/Akt (Marchetti et al., 2004; Senechal et al., 2008). *In vitro* this PKB/Akt pathway was also shown to underlie the lovastatin-mediated protection against glutamate-induced excitotoxicity (Dolga et al., 2008). To investigate if this is also the case in the *in vivo* situation, we used the inhibitor of PI3 kinase, LY294002, to block the activation of PKB/Akt. In the presently used experimental setup this LY294002 treatment resulted in a strong reduction of lovastatin-mediated neuroprotection *in vivo*.

The neuroprotective effect of PKB/Akt activation was previously reported in *in vivo* models for brain injury. In a mid-cerebral artery occlusion model (Fontaine et al., 2002; Prinz et al., 2008) PKB/Akt activation was found to be essential for neuroprotective signaling since neuroprotection after post-conditioning was inhibited in the presence of the PKB/Akt inhibitor LY294002. Besides suppressing the PI3 kinase activity, LY294002 also has the capacity to block casein kinase 2. However casein kinase 2 in turn uses phosphatase and tensin homolog deleted on chromosome 10 (PTEN) as major substrate which will also lead in the end to inhibition of the PKB/Akt signaling pathway (Duncan and Litchfield, 2008).

Our findings corroborate recently reported microarray and RT-PCR studies that identified several genes of which the expression was altered in statin-treated mice. Lovastatin treatment increased the expression levels of 26 genes particularly genes related to apoptosis (*c-myc*, *Bcl2*) (Johnson-Anuna et al., 2005) and PKB/Akt phosphorylation (Li et al., 2006). Bcl2 proteins exhibit neuroprotective functions against various excitotoxic insults, such as glutamate or amyloid beta peptides. *Bcl2* gene expression is of particular interest in this respect since this major anti-apoptotic gene is under direct PKB/Akt regulation (Pugazhenthi et al., 2000).

In order to assess whether the cholinergic neurons rescued by lovastatin treatment are able to counteract the behavioral deficits observed in NMDA-induced lesion mice we subjected the animals to a spontaneous alternation task and a passive avoidance memory test. To test the selectivity of the MNB lesion to cortical functions we used the spontaneous alternation paradigm for working memory, since this test is commonly associated with hippocampus and pre-frontal-related memory. We found no significant difference in alternation rates between NMDA-lesioned animals compared with non-lesioned control mice. This suggests that brain regions as the hippocampus are not affected by NMDA-induced lesions of MNB, which is in line with the known projection patterns of the MNB and medial septum complex (Gaykema et al., 1989; Luiten et al., 1987). On the other hand, pre-lesion administration of lovastatin significantly attenuated the memory retention deficit induced by the NMDA lesion of the MNB. The passive avoidance paradigm is one of the test conditions of choice for the behavioral assessment of the integrity of the neocortical system. Thus, the neuroprotective effect of lovastatin on NMDA-induced damage to the MNB and its neocortical innervation are in line with the improved memory retention in lovastatin treated mice. In summary, our behavioral data indicate impaired behavioral performance associated with damage to the MNB that specifically affects neocortical denervation and its memory functions while leaving particular hippocampal innervation and its learning functions unaffected. Whereas lovastatin treatment reverted the memory deficits produced by NMDA lesions as observed in the passive avoidance paradigm, lovastatin treatment itself had a modest albeit significant effect on improving hippocampal memory and prefrontal cortex function irrespective of damage to cholinergic neocortical innervation. In a recently published paper the effect of simvastatin on hippocampal long-term potentiation (LTP) in brain slices was reported, showing that simvastatin inhibits LTP, most likely by inhibiting the production of geranylgeraniol. In addition, inhibition of mevalonate pathways produced deficiencies in spatial, associative and motor learning. This indicates that cholesterol by itself might be important for learning and memory functions, but more critical are the byproducts from the mevalonate pathway, such as geranylgeranyl pyrophosphate (Kotti et al., 2006).

It is unlikely that behavioral alteration of lovastatin-treated animals can be attributed to direct drug effect since statins are rapidly cleared from the brain within 6 hours after drug administration (Johnson-Anuna et al., 2005). We performed all behavioral testing 7 days after the last lovastatin administration. However, the exact underlying mechanisms of how statins can affect the brain remain to be elucidated (Wolozin, 2004). Statins such as lovastatin are able to cross the blood brain barrier and reach the cerebral cortex within one hour after drug administration but are significantly reduced 6 hours later after a single drug application. It is not clear whether the effects of statins as found in the present study can be directly associated with cholesterol dependent processes in brain tissue. Brain cholesterol analyses in several studies showed conflicting results. Long-term treatment of simvastatin for 3 months did not change the total cholesterol levels in the brain although the plasma total cholesterol levels were strongly reduced (Li et

al., 2006). Other studies report reduced brain cholesterol levels in young C57Bl/6J mice treated for 3 weeks with simvastatin and pravastatin, but such a decline of cholesterol concentrations was not observed in the brains of mice treated with lovastatin (Johnson-Anuna et al., 2005).

In conclusion our data provide evidence for a neuroprotective role of lovastatin against NMDA-induced lesions of the MNB. Importantly, this beneficial effect was shown to be dependent on activation of the PKB/Akt pathway. Furthermore, lovastatin administration was able to attenuate impaired memory functions that can be associated with cholinergic cortical denervation that result from the damaged nucleus basalis-cortical projection pathway.

CHAPTER 3

TNF-α mediates neuroprotection against glutamate -induced excitotoxicity via NF-κB-dependent up-regulation of KCa2.2 channels

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ABSTRACT

Previous studies have shown that tumor necrosis factor-alpha (TNF-α) induces neuroprotection against excitotoxic damage in primary cortical neurons via sustained nuclear factor-kappa B (NF-κB) activation. The transcription factor NF-κB can regulate the expression of small conductance calcium-activated potassium (KCa) channels. These channels reduce neuronal excitability and as such may yield neuroprotection against neuronal overstimulation. In the present study we investigated whether TNF-α-mediated neuroprotective signalling is inducing changes in the expression of small conductance KCa channels. Interestingly, the expression of KCa2.2 channel was up-regulated by TNF-α treatment in a time-dependent manner whereas the expression of KCa2.1 and KCa2.3 channels was not altered. The increase in KCa2.2 channel expression after TNF-α treatment was shown to be dependent on TNFR2 and NF-κB activation. Furthermore, activation of small conductance KCa channels by NS309 or CyPPA induced neuroprotection against a glutamate challenge. Treatment with the small conductance KCa channel blocker apamin or KCa2.2 channel siRNA reverted the neuroprotective effect elicited by TNF-α. We conclude that treatment of primary cortical neurons with TNF-α leads to increased KCa2.2 channel expression which renders neurons more resistant to excitotoxic cell death.

3.1 INTRODUCTION

TNF-α is a proinflammatory cytokine involved in a wide range of cellular responses including inflammation, cellular differentiation and apoptosis. TNF-α per se can be synthesized and released in the brain by several types of cells, including astrocytes, microglia, and neurons. TNF-α was shown to be up-regulated in a number of neurodegenerative disorders including Alzheimer's disease, stroke, and multiple sclerosis. It exerts its biological functions through the stimulation of two receptors, TNFR1 and TNFR2. Both TNFR1 and TNFR2 induce activation of the NF-κB pathway (Eisel et al., 2006; Marchetti et al., 2004). However, the activation kinetics of NF-κB stimulation reveals characteristic differences: while activation of the TNFR1 pathway leads to a transient NF-κB stimulation, TNFR2 signalling promotes persistent and long lasting NF-κB activation (activation that even persists up to 24 h of TNF-α treatment)(Marchetti et al., 2004).
Several studies showed that TNF-α is able to promote neuroprotection against glutamate-induced excitotoxicity in cortical neurons (Cheng et al., 1994; Marchetti et al., 2004). Until now, the molecular mechanisms underlying the neuroprotective effects of TNF-α against glutamate-induced excitotoxicity are largely unknown but at least PKB/Akt activation and subsequent sustained NF-κB activation were shown to play a major role (Marchetti et al., 2004).

In a recent study, small conductance Ca^{2+} activated potassium (KCa2.1-3) channels were identified as one of the downstream targets of NF-κB mediated promoter regulation (Kye et al., 2007). Together with intermediate conductance KCa channels (KCa3.1), these small conductance KCa channels form one of the two subgroups of KCa channels (Wei et al., 2005). They differ from the other subgroup (KCa1.1, KCa4.1/4.2 and KCa5.1 channels) in that they are voltage insensitive, and open in response to low (< 1 mM) intracellular Ca^{2+} levels by virtue of calmodulin, which is constitutively bound to the C-terminal region. The small conductance KCa channels are sensitive to apamin, which distinguishes them from all other KCa channels. Small conductance KCa channels are important regulators of neuronal excitability. In many central nervous system neurons, small conductance KCa channel activity dampens excitability by contributing to the afterhyperpolarization, affecting interspike intervals during a burst of action potentials as well as the length of the burst (Stocker, 2004). In the mammalian brain small conductance KCa channels are the product of three paralogous genes, namely KCNN1, KCNN2 and KCNN3 genes. KCa2.1 and KCa2.2 channel subunits show extensive co-localization. They are mainly found in the entorhinal cortex, the subiculum, in pyramidal cortical neurons, the CA1 - CA3 region of the hippocampus and in the thalamus. KCa2.3 channel subunits have a complementary distribution, and are mainly located in the brain stem and in monoaminergic neurons (Sailer et al., 2002; Sailer et al., 2004). In certain neuronal cell types, small conductance KCa channels are present at dendritic spines where they co-localise with NMDA receptors. There, small conductance KCa channel activity suppresses the amplitude of evoked synaptic potentials by inhibiting NMDA receptor-dependent activation (Faber et al.,

2005; Lin et al., 2008; Ngo-Anh et al., 2005). Thus, enhancing small conductance KCa channel expression during an excitotoxic insult could be protective due to hyper-polarization and diminished excitability. In an initial study Lee and colleagues showed indeed that overexpression of KCa2.2 channels can prevent kainic acid- and glutamate-induced excitotoxicity (Lee et al., 2003).
In the present study we have tested the hypothesis whether increased small conductance KCa channel expression is part of the mechanism of TNF-α mediated neuroprotection against glutamate-induced excitotoxity in cortical neurons.

3.2 MATERIAL AND METHODS

3.2.1 Materials

Neurobasal medium and B27 supplement were purchased from Invitrogen (Carlsbad, CA, USA). Complete mini protease inhibitor cocktail tablets were from Roche (Indianapolis, IN) and mouse TNF-α was a gift from HBT (Uden, The Netherlands) or was purchased from Peprotech (London, UK). Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methylpyrimidin- 4-yl]-amine (CyPPA) was bought from Specs (Delft, Netherlands). Primary antibodies used to detect small conductance KCa channels were rabbit anti-KCa2.1, rabbit anti-KCa2.2 (both kindly provided by H-G. Knaus, (Sailer et al., 2004)), and anti-KCa2.3 (APC-025; Alomone Laboratories, Jerusalem, Israel). For the detection of actin a monoclonal mouse antibody from MP Biomedicals (691001 Irvine, CA, USA) was used. Alkaline phosphatase (AP)-conjugated goat anti-mouse (Applied Biosystems, Bedford, MA, USA) and AP-conjugated goat anti-rabbit (Applied Biosystems, Bedford, MA, USA) were used as secondary antibodies. The chemi-luminiscence detection kit (Nitroblock II and CDP-Star) was purchased from Applied Biosystems (Bedford, MA, USA). All other materials were from Sigma.

3.2.2 Animal experiments

All experiments were performed using C57BL/6J (Harlan, Horst, The Netherlands), TNFR1-/- or TNFR2-/- mice (Erickson et al., 1994; Pfeffer et al., 1993; Rothe et al., 1993). TNFR1-/- or TNFR2-/- mice were kept in a C57BL/6J background for more then ten generations. All procedures were in accordance with the regulation of the ethical committee for the use of experimental animals of the University of Groningen, The Netherlands (Licence number DEC 4048). Mice were housed in standard macrolon cages and maintained on a 12 h light/dark cycle. They received food and water ad libitum.

3.2.3 Primary cortical neuron culture

Primary cortical neurons were prepared from embryonic brains (E14-16) of C57Bl/6J, TNFR1-/- or TNFR2-/- mice. The meninges were removed and the cortical neurons were separated by mechanical dissociation. Cells were plated in a density of 12 x 10^4 cells/well (96 well plates) and 2 x 10^6 cells/well (6 well plates) on poly-D-lysine pre-coated plates. Neurobasal medium with 2% (v/v) B27-supplement, 0.5 mM glutamine, 1% (v/v) penicillin/ streptomycin and 2.5 mg/ml amphotericin B was used as a culture medium. After 48 h neurons were treated with 10 mM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and after 6 days of in vitro culture, the neurons were used for experiments. Neurons were incubated for the indicated periods of time with 100 ng/ml mouse TNF-α. Some cortical neurons were incubated with the NF-κB inhibitor BAY11-7082, the small conductance KCa channel activator NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) or CyPPA or the small conductance KCa channel blocker apamin for the indicated time period and concentrations. BAY11-7082, NS309 and CyPPA were prepared as stock solution in DMSO. The final concentration of DMSO in all experiments was less than 0.5% vol/vol. In this concentration DMSO had no effect on glutamate-induced cell death (data not shown). Apamin was prepared as stock solution in toxin buffer (100 mM NaCl, 20 mM Hepes pH = 7.4) and kept at -20° C until use. Excitotoxicity was induced by 1 h treatment with glutamate (50 mM). After treatment, the medium was completely removed and fresh medium was added to the cells.

3.2.4 Determination of cell viability

24 h after glutamate treatment neuronal viability was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide) assay as described previously (Mosmann, 1983). 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2-4 h of incubation, cells were lysed by adding 120 ml of isopropyl-HCl solution (37% HCl/isopropyl alcohol: 1/166) for 15 min. The absorbance of each well was determined with an automated ELISA reader (Bio-Rad, Munich, Germany) at 595 nm with a reference filter at 630 nm.

3.2.5 Protein analysis

Primary cortical neurons from C57Bl/6J, TNFR1-/- or TNFR2-/- mice were washed twice with ice cold PBS after indicated periods of time of TNF-α incubation and subsequently lysed by the addition of 0.15 ml lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and complete mini protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)). The samples were centrifuged at 9,000g for 10 min at 4° C and the supernatants boiled for 5 min in Laemmli's sample buffer. Twenty mg of total protein was separated by 10% SDS-polyacrylamide gel

electrophoresis. Proteins were transfered to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). Overnight the membranes were incubated with primary antibodies at 4° C and afterwards with AP-conjugated secondary antibodies (1: 10,000). Proteins were detected with an ECL detection system according to the manufacturer's instructions (Applied Biosystems, Bedford, MA, USA). Actin (1:100,000 antibody dilution) was used as an internal control to correct for variations in protein content. The level of actin was not affected by TNF-α treatment. Integrated optical densities (IOD) were measured with the Leica DFC 320 Image Analysis System (Leica, Cambridge, UK) and densitometric analysis was performed using the Leica Qwin program. For quantitative image analysis we determined the histogram profile of each band and the peaks corresponding to the immunoreactive area were measured (IOD). Quantification of IOD was done only with images in which saturation of the signal had not occurred. IOD measurements were corrected for background intensity.

3.2.6 Subcellular fractionation

Primary cortical neurons cultured in 6-well plates were lysed in lysis buffer (50 mM Tris- HCl pH 7.4, 12.5 mM EDTA, 12.5 mM EGTA, 400 mM sucrose, 0.3% (v/v) β- mercaptoethanol, complete mini protease inhibitor cocktail tablet). In order to obtain the cytosolic and membrane fraction, the subcellular components were separated by a series of centrifugations. In a first centrifugation step at 2,000g for 2 min cell debris and nuclei were removed. In a second centrifugation step the supernatant was centrifuged at 100,000g for 60 min. The supernatant of this centrifugation step contained the cytosolic fraction whereas the pellet fraction was referred to as membrane fraction. The pellet was resuspended in 50 ml lysis buffer.

3.2.7 siRNA experiments

siRNA probes targeted to KCa2.2 channels were purchased from Dharmacon (Dharmacon, Inc. Lafayette, CO, USA). The target sequences for the mouse-specific KCa2.2 channel Accell SMARTpool siRNA mixture were as follows: CUAGAGACUUUGAUUGGUA (A-049792-13), CGAUCAUCCUGCUUGGUCU (A-049792-14), GUAUUUUCUUCAUCU GCUU (A-049792-15), CGUUUUGUUAUGAAGACUU (A- 049792-16).
A non-targeting Accell siRNA pool (D-001910-10) was used as a control in all siRNA transfection experiments. Primary cortical neurons were transfected with Accell siRNAs in Accell delivery media (B-005000) according to the manufacturer's instructions. Primary cortical neurons grown on 96-well plates were treated with Accell siRNA probes after 4 days. Cells were cultured for 3 more days after transfection, then treated, lysed and subsequently analyzed for protein content. Accell siRNA probes were in the medium when TNF-α was applied to neurons.

3.2.8 Statistical analysis

Each experiment was performed with at least two different batches of neurons and MTT and Western blot were repeated at least twice for each batch. Results are represented as mean ± SEM. Statistical analysis was performed by the Student-Neumann test with a 95% confidence interval followed by one-way ANOVA post-hoc LSD and Dunnett test using the SPSS program. P values of <0.05 were considered to be significant.

3.3 RESULTS

3.3.1 TNF- α protects neurons against glutamate-induced excitotoxicity

To assess the time-dependent effect of TNF-α pre-treatment on cortical neuronal cultures challenged with glutamate, we exposed cortical neurons to mouse TNF-α (100 ng/ml) for 2, 4, 6, 8 or 24 h. After incubation with TNF-α, cortical neurons were challenged with glutamate (50 mM, for 1 h). Neuronal viability was determined 24 h following glutamate exposure by the colorimetric MTT assay. Glutamate treatment alone reduced cell survival to 36.4 ± 3.2% when compared to untreated neurons (set to 100%). Pre-incubation of neurons with TNF-α for up to 6 h had no significant protective effect against glutamate induced excitotoxicity (Figure 3.1). The number of surviving neurons gradually increased when the TNF-α pre-treatment lasted for more than 6 h up to 62.2 ± 6.3% cell survival after 24 h TNF-α treatment. Treatment with TNF-α (100 ng/ml) alone for 24 h did not change neuronal viability (Figure 3.1). Furthermore, TNF-α treatment after the glutamate challenge did not protect cortical neurons against glutamate-induced toxicity (data not shown).

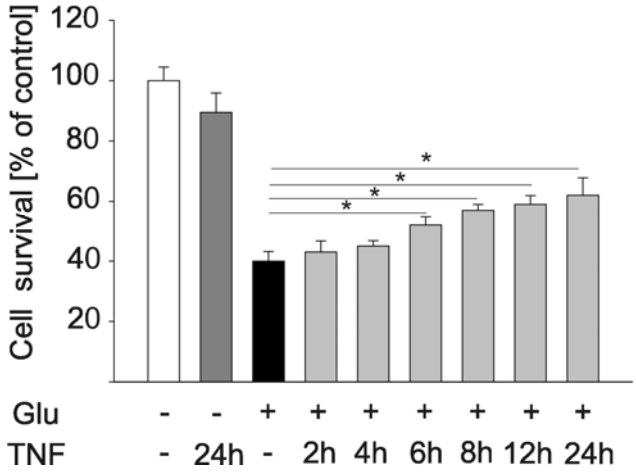

Figure 3.1. *TNF-α -mediated neuroprotection against glutamate-induced excitotoxicity. Cortical neurons were treated with mouse TNF-α (100 ng/ml) for the indicated periods of time and subsequently challenged with glutamate (50 mM, 1 h). Neuronal survival was assessed 24 h following the exposure to glutamate by an MTT assay. Values represent mean ± SEM (n = 6). Statistical analysis was performed by the Student-Neumann test with a 95% confidence interval using the SPSS program. *P values of <0.05 were considered to be significant.*

3.3.2 TNF-α increases KCa2.2 channel expression

Activation of small conductance KCa channels in cortical neurons has been shown to suppress hyperexcitability (Pedarzani et al., 2001). This could be a mechanism to enhance neuronal survival in conditions of overstimulation. Therefore, we determined whether TNF-α treatment leads to a change in the expression of the three small conductance KCa channels, KCa2.1, KCa2.2 or KCa2.3. Similar to a previous study in mouse brain (Sailer et al., 2004), we found that anti-KCa2.1(12-29) antibody (1:1,000) detects three separate bands with molecular weights of 65, 58 and 43 kDa. The affinity-purified anti-KCa2.2(538-555) antibody (1:1,000) detects a band with an apparent Mr value of 64 kDa whereas the serum antibody showed an additional unspecific band at approx. 67 kDa. The anti-KCa2.3(504- 522) antibody (1:1,000) recognizes a protein running at the height of 75 kDa. Glutamate treatment did not affect the expression of any of the small conductance KCa channels (data not shown), whereas treatment of cortical neurons with TNF-α (100 ng/ml) for 2, 4, 6, 8 and 24 h had no effect on the expression of KCa2.1 and KCa2.3 channels (Figure 3.2A, D).

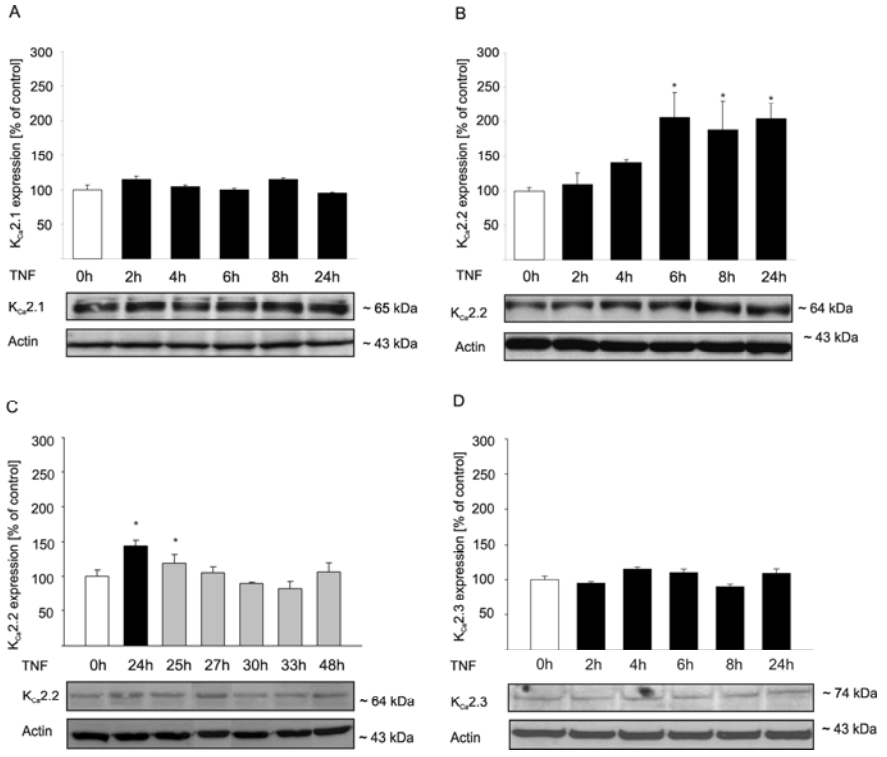

Figure 3.2. *Exposure to TNF-α increases KCa2.2 channel expression in cortical neurons. Neurons were treated with mouse TNF-α (100 ng/ml) for indicated periods of time. A. Representative blot and bar graph showing the quantification of the 65 kDa KCa2.1 channel band. Accordingly, integrated optical densities for the other two KCa2.1 channel bands at 58 and 43 kDa showed no changes in response to the indicated TNF-α incubation times. B. Quantification and representative immunoblot for KCa2.2 channels with apparent Mr of 64 kDa after exposure to TNF-α for indicated periods of time. C. Quantification and expression of KCa2.2 channels after TNF- α (24 h) was removed from thee media. D. Quantification and representative immunoblot for KCa2.3 channels. Results shown represent mean ± SEM (n = 4). Statistical analysis was performed by the Students-t test or Student-Neumann test with a 95% confidence interval followed by one-way ANOVA post-hoc LSD and Dunnett test using the SPSS program. *P values of <0.05 were considered to be significant.*

Shorter incubation of TNF-α also showed no effect on KCa2.2 channel expression when compared to untreated neurons. However, KCa2.2 channel expression increased significantly when neurons were treated with TNF-α for 6, 8, and 24 h (24 h TNF-α: 204.6 ± 22.1% of non-treated neurons; Figure 3.2B). KCa2.2 channel expression returned within 3h to basal levels, when the medium was replaced with TNF-α free medium (Figure 3.2C). Subcellular fractioning revealed that under basal conditions KCa2.2 channels are located both in the cytosolic and membrane fraction but they are targeted to the membrane upon TNF-α treatment (Figure 3.3).

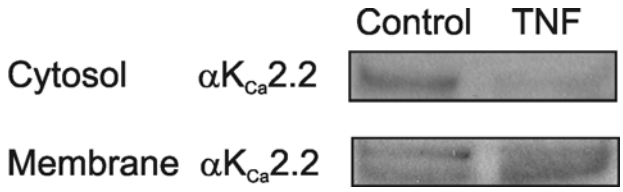

Figure 3.3. *TNF-α treatment increases KCa2.2 channel immunoreactivity. Representative blot of KCa2.2 channel in the cytosolic and membrane fraction in non treated neurons and after 24 h 100 ng/ml mouse TNF-α treatment (n = 4).*

3.3.3 TNF-α-induced upregulation of KCa2.2 channels is mediated by TNFR2

TNF-α initiates signaling cascades by binding to its receptors: TNFR1 and TNFR2 (Eisel et al., 2006). To investigate which of the two receptors is involved in the upregulation of KCa2.2 channels after prolonged TNF-α treatment, we isolated neurons from TNFR1-/- or TNFR2-/- mouse embryo brains. Interestingly, we could only observe increased KCa2.2 channel expression after TNF-α treatment in TNFR1-/- primary neurons (492.7 ± 13.7%), whereas the level in TNFR2-/- was unchanged (Figure 3.4).

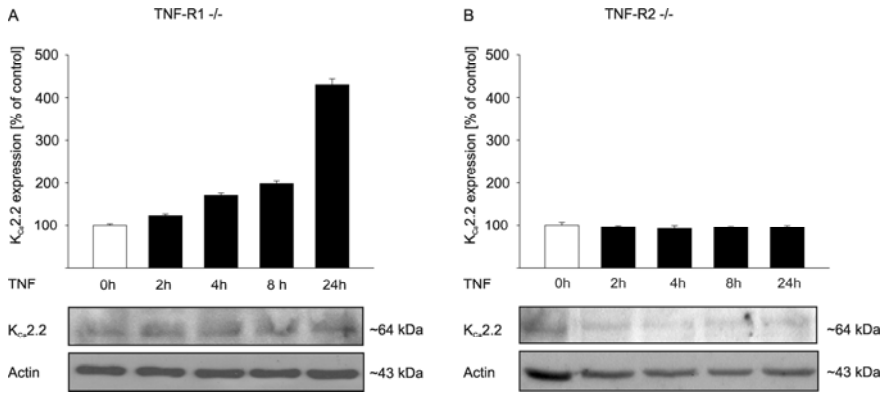

Figure 3.4. *The TNF-α dependent increase in KCa2.2 channel is dependent on TNF-R2. Primary cortical neurons from (A) TNF-R1-/- and (B) TNF-R2-/- mice were incubated with 100 ng/ml mouse TNF-α for 2-24 h, lysed and KCa2.2 channel expression assessed using Western blotting. Results shown represent mean ± SEM (n = 4). Statistical analysis was performed by the Student's-t test with a 95% confidence interval using the SPSS program. *P values of <0.05 versus non-treated neurons were considered to be significant.*

3.3.4 Inhibition of NF-κB attenuates TNF-α induced upregulation of KCa2.2 channel expression

The activation kinetics of the transcription factor NF-κB in response to TNF-α treatment in neurons was shown by several groups (Kaltschmidt et al., 1999; Marchetti et al., 2004; Wang et al., 2005). It has been reported that long-term (24 h) exposure of neurons to TNF-α promotes persistent and long lasting NF-κB activation, which is associated with increased protection against glutamate-induced neurotoxicity (Marchetti et al., 2004). We confirmed these data in our experimental set-up showing that inhibition of NF-κB by a specific inhibitor, BAY11-7082 (10 mM), reversed the TNF-α-mediated neuroprotective effect (Figure 3.5A). Since KCa2.2 channel expression is regulated by NF-κB (Kye et al., 2007), we determined whether the observed increase in KCa2.2 channel expression after TNF-α incubation was NF-κB dependent. KCa2.2 protein expression was evaluated by Western blot analyses in TNF-α treated neurons in the presence or absence of BAY11-7082. KCa2.2 levels in cortical neurons were not increased by application of TNF-α (100 ng/ml) when BAY11-7082 (10 mM) was co-applied (Figure 3.5B). BAY11-7082 treatment alone did not affect KCa2.2 channel expression. These findings suggest that inhibition of NF-κB prevented the TNF-α-induced increase in KCa2.2 channel expression (Figure 3.5B).

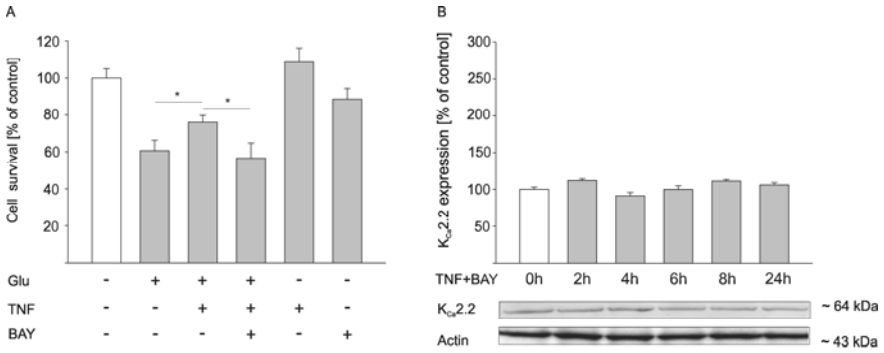

Figure 3.5. *NF-kB inhibition prevents the TNF-α-induced neuroprotection and the increase in KCa2.2 channel expression A. Neuronal survival was assessed after 24 h TNF-α (100 ng/ml) incubation followed by an 1 h glutamate treatment. BAY11-7082 (10 mM) was co-applied with TNF-α where indicated. B.Neurons were co-incubated with TNF-α and BAY11-7082 for the indicated periods of time. Quantification and a representative immunoblot for KCa2.2 channel protein with apparent Mr values detected at 64 kDa are presented. Results shown represent mean ± SEM (n = 3). Statistical analysis was performed by the Student- Neumann test with a 95% confidence interval followed by one-way ANOVA post-hoc LSD and Dunnett test using the SPSS program.*

3.3.5 Small conductance KCa channel activation leads to neuronal survival

To investigate whether increased small conductance KCa channel activity can be neuroprotective, cortical neurons were treated for 30 min with different concentrations of NS309, an activator of small conductance KCa and intermediate conductance KCa (KCa 3.1) channels (Strobaek et al., 2004), followed by addition of glutamate (50 mM) to the medium for 1 h. NS309 strongly reduced glutamate-induced neuronal death in a concentration-dependent manner (cell survival NS309 100 mM and glutamate 86.8 ± 1.8% versus glutamate 46.7 ± 5%)(Figure 3.6A). In order to have neuroprotective properties NS309 had to be in the medium during the glutamate challenge. When NS309 was given before or after the glutamate challenge, we could not observe a neuroprotective effect of the KCa channel activator (Figure 3.6B).

Since NS309 does not distinguish between the subtypes KCa2.1, KCa2.2 and KCa2.3 and also activates the related KCa3.1 channels, we repeated this experiment with CyPPA, a compound which is selective for stimulating KCa2.2 and KCa2.3 (Hougaard et al., 2007). Treatment with CyPPA also led to increased neuronal survival upon glutamate treatment in a concentration-dependent manner although to a somewhat lower extent as NS309 (cell survival CyPPA 50 mM and glutamate 52.4 ± 3.1% versus glutamate 36.9 ± 3.3%)(Figure 3.6C). Concentrations of CyPPA higher than 50 mM, however, appeared to be toxic to neurons.

3.3.6 Reduced KCa 2.2 channel activity blocks TNF-α-induced neuroprotection

To confirm the involvement of small conductance KCa channels in TNF-α-induced neuroprotection, we analyzed the viability of cortical neurons that were treated with 100 ng/ml mouse TNF-α for 24 h and challenged with glutamate (50 mM for 1 h) in the presence or absence of an inhibitor of small conductance KCa channel activity (Figure 3.6D). As inhibitor we used apamin, an 18-amino-acid bee-venom toxin with a high selectivity for small conductance KCa channels. Application of apamin alone in a concentration of 50 mM already reduced cell survival to 73.3 ± 2.7% whereas a concentration of 20 mM apamin had no significant effect on cell survival. The detrimental effect of glutamate on cell survival was also not changed by 20 mM apamin treatment. Glutamate-induced neuronal death was partially reverted by pre-incubation of TNF-α (TNF-α and glutamate 56.7 ± 2.7% cell survival versus glutamate 41.2 ± 1.5%), whereas suppression of small conductance KCa channel activity by apamin (20 mM) prevented the TNF-α induced neuroprotective effect (TNF-α, apamin and glutamate 42 ± 1.3% cell survival versus glutamate 41.2 ± 1.5%) (Figure 3.6D).

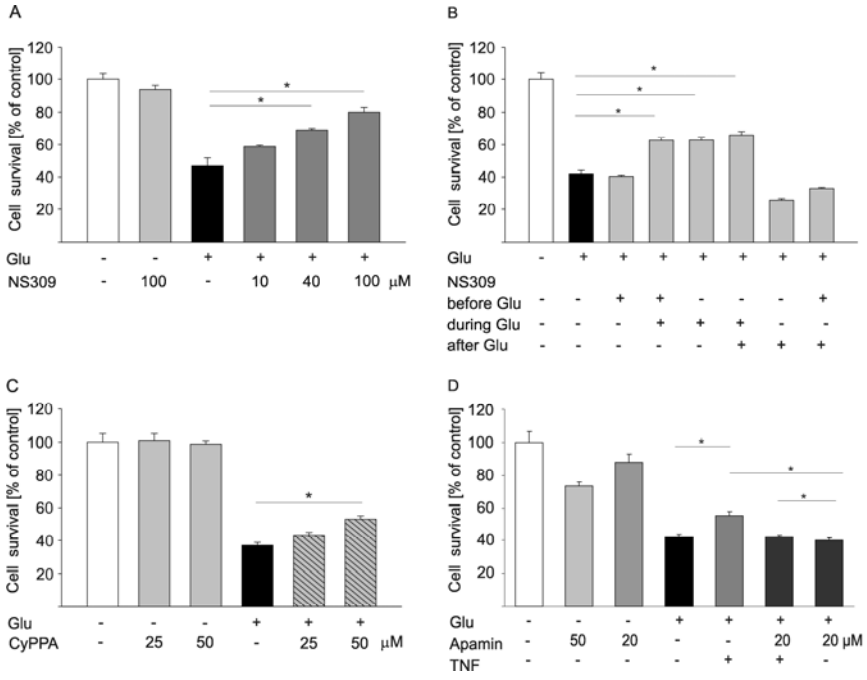

Figure 3.6. *Small conductance KCa channel activation induces neuroprotection A. Cortical neurons were incubated with the small conductance KCa channel activator, NS309 (10 - 100 mM) for 30 min. Then, where indicated, neurons where challenged by adding glutamate (50 mM) to the medium for 1 h. After the glutamate challenge, media were completely replaced by fresh media. After an additional 24 h the viability of cells was verified by an MTT assay. B. NS309 was added to the neurons 30 min before, during and/or immediately after the glutamate challenge (50 mM, 1 h) and cell survival assessed by an MTT assay. C. CyPPA (25 mM and 50 mM), a positive modulator of KCa2 channels with a high selectivity for KCa2.3 and KCa2.2 channels, was applied alone for 30 min or the 30 min treatment was followed by adding glutamate to the medium for 1h where indicated. D. Cortical neurons were treated with TNF-α for 24 h. Afterwards neurons were challenged with glutamate (50 mM) for 1 h. Where indicated, the small conductance KCa channel blocker apamin was added to the medium for 30 min before adding glutamate to the medium. Statistical analysis was performed by the Student-Neumann test with a 95% confidence interval. *P values of <0.05 versus glutamate treated neurons were considered to be significant (n = 3).*

The role of KCa2.2 channels in TNF-α- induced neuroprotection was assessed by pre-treating primary cortical neurons with KCa2.2 channel siRNA. Protein analysis of neurons pre-treated with KCa2.2 channel siRNA showed reduced KCa2.2 channel expression levels compared to non-treated neurons or control siRNA-treated neurons. Most important, the up-regulation of KCa2.2 channel expression levels observed after TNF-α treatment was abolished when neurons were pre-treated with KCa2.2 channel siRNA but not when pre-treated with control siRNA (Figure 3.7A). Treatment with KCa2.2 channel siRNA alone already significantly reduced

the viability of the neurons (80.1 ± 2.2%) (Figure 3.7B). TNF-α-induced neuroprotection against a glutamate challenge was completely abolished when neurons were pre-treated with KCa2.2 channel siRNA. In control siRNA-treated neurons TNF-α still induced protective effects against glutamate excitotoxicity (Figure 3.7B).

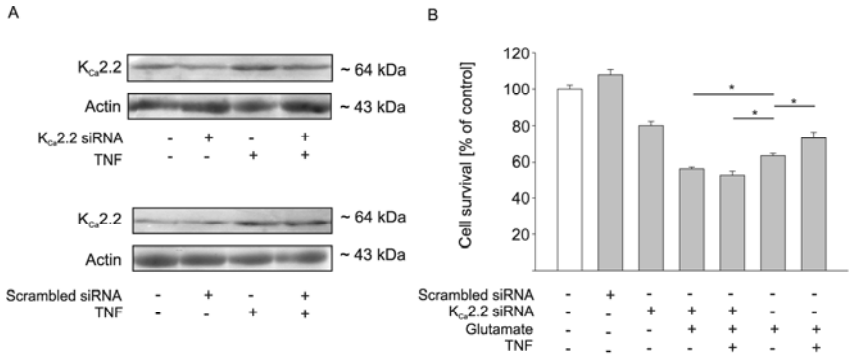

Figure 3.7. *KCa2.2 channel siRNA pre-treatment abolishes TNF-α-induced neuroprotection Cortical neurons were pre-treated with KCa2.2 siRNA or control siRNA. After 2 days of incubation with siRNA, 100 ng/ml TNF-α was added to the medium for 24 h. Neurons which were not treated with TNF-α served as controls. A. Representative blots of KCa2.2 channel expression. B. Neuronal survival assessed by an MTT assay. TNF-α was applied for 24 h and followed by an 1 h exposure to 50 mM glutamate where indicated. Values represent mean ± SEM (n = 6). Statistical analysis was performed by the Student- Neumann test with a 95% confidence interval using the SPSS program. *P values of <0.05 versus glutamate were considered to be significant.*

3.4 DISCUSSION

In the present study we have demonstrated that a TNFR2 and NF-κB-dependent increase in KCa2.2 channel expression in cortical neurons contributes to the neuroprotective effect of TNF-α against glutamate-induced excitotoxicity. TNF-α has been implicated as contributing to both neuroprotection and neurodegeneration, depending on the tissue and experimental paradigm (Chao and Hu, 1994; Cheng et al., 1994; Hermann et al., 2001; Marchetti et al., 2004; Zou and Crews, 2005). In our in vitro model of glutamate-induced cell death of primary cortical neurons, TNF-α was shown to have neuroprotective properties. Although the involvement of TNFR2, PKB/Akt and NF-κB was already suggested (Dolga et al., 2008; Fontaine et al., 2002; Marchetti et al., 2004), only limited data are available on potential neuroprotective downstream targets of NF-κB. In our study, the time-dependent neuroprotective effect of TNF-α against a glutamate challenge was paralleled by a selective increase in KCa2.2 channels, whereas the expression of KCa2.1 and

KCa2.3 channels remained unaffected. More specifically, TNF-α treatment resulted in a translocation and increased expression of KCa2.2 channels at the cell surface. The increased expression of KCa2.2 channels after TNF-α treatment was mediated by TNFR2, which corresponds well with our previous findings that TNFR2 mediates TNF- α-induced neuroprotection (Dolga et al., 2008; Fontaine et al., 2002; Marchetti et al., 2004). In addition, NF-κB was shown to be involved in the enhanced expression of KCa2.2 channels after TNF-α treatment. BAY11-7082, a specific inhibitor of NF-κB, blocked the TNF-α mediated increase in KCa2.2 channel levels and was also shown to block TNF- α/TNFR2 mediated neuroprotection (Marchetti et al., 2004). NF-κB might interact with two NF-κB binding sites, which were recently reported as regulatory parts of the murine KCa2.2 promoter (Kye et al., 2007). The specific small conductance KCa channel blocker apamin blocked the neuroprotective effect of TNF-α against glutamate-induced excitotoxicity. Small conductance KCa channels have different affinities for apamin with KCa2.2 proteins being the most sensitive, KCa2.3 intermediate and KCa2.1 being the least sensitive to apamin (Finlayson et al., 2001; Strobaek et al., 2000). Given the finding that in cortical neurons KCa2.1 and KCa2.2 channels are more abundant than KCa2.3 channels (Sailer et al., 2002; Sailer et al., 2004) and KCa2.2 channels are more sensitive to apamin than KCa2.1 channels (D'Hoedt et al., 2004), reduction of TNF-α mediated neuroprotection by apamin could be mainly attributed to the inhibition of KCa2.2 channels. This was confirmed by our findings using KCa2.2 channel siRNA. Reduced KCa2.2 channel expression after KCa2.2 channel siRNA treatment abolished TNF-α mediated neuroprotection. The neuroprotective function of small conductance KCa channels became evident by the finding that the small conductance KCa channel activators, NS309 and CyPPA, induced neuroprotection against glutamate-induced excitotoxicity in primary cortical neurons.

NS309 facilitates Ca^{2+}-dependent activation of small and intermediate conductance KCa channels, being most potent on intermediate KCa channels and 10–20 times less active on the small conductance KCa channel subtypes (Strobaek et al., 2004). CyPPA is a selective positive modulator of KCa2.2 and KCa2.3 channels, which does not affect KCa2.1 or KCa3.1 channels (Hougaard et al., 2007). Although we can not exclude the involvement of KCa2.3 channel activity in the neuroprotective effect elicited by CyPPA, the distribution profile of KCa2.2 and KCa2.3 in cortical neurons (Sailer et al., 2002) again suggests a major role of KCa2.2 in the CyPPA-induced neuroprotective effect. The neuroprotective effect of CyPPA was similar to the neuroprotective effect of TNF-α whereas the effect of NS309 was much larger. This result may be explained by a possible neuroprotective effect of KCa3.1 channel activation in NS309 treated-neurons. Based on our results we can only speculate on how increased KCa2.2 channel expression can contribute to the neuroprotective effect elicited by TNF-α. However a KCa2.2-mediated decrease in neuronal excitability is likely to be part of the mechanism. Indeed, the neuroprotective potential of KCa2.2 has also been observed in an earlier study showing that overexpression of KCa2.2 channels in cultured hippocampal neurons increases cellular survival against a kainic acid challenge by blunting the kainic acid-induced

increase in excitability (Lee et al., 2003). Several studies showed a colocalization of KCa2.2 channels with NMDA receptor subunits in dendritic spines (Faber et al., 2005; Lin et al., 2008; Ngo-Anh et al., 2005). Glutamate treatment of neurons triggers ionotropic glutamate (e.g. NMDA, AMPA) receptor dependent activation which leads to excessive Ca^{2+} influx into the cell. KCa2.2 channels can be activated by this Ca^{2+} influx. It was shown that the repolarizing effect of KCa2.2 channel activity can oppose the depolarizing effect of AMPA receptor activity, favouring Mg^{2+} reblocking of NMDA receptors and thus reducing the Ca^{2+} transient (Faber et al., 2005; Lin et al., 2008; Ngo-Anh et al., 2005). In this way KCa2.2 channels may decrease glutamate-induced excitotoxicity. We hypothesize that increased KCa2.2 channels expression/activity may lead to decreased susceptibility to glutamate-induced excitotoxicity via its coupling with NMDA receptors. However, based on the results by Houzen and colleagues (Houzen et al., 1997), it can be suggested that an increase in KCa2.2 channels is not the only mode of neuroprotection against glutamate-induced excitotoxicity. They reported that the neuroprotective effect of TNF-α against NMDA-induced toxicity can be attributed to an increase in outward potassium current (A-current) density in rat cortical neurons. From these findings, it may be concluded that the neuroprotective effect of TNF-α against glutamate-induced toxicity is mediated by a concomitant increase in outward potassium current (A-current) density. The A-current is shown to be involved in the regulation of the neuronal firing rates in such a way that an increase in A-current density reduces cell excitability (Houzen et al., 1997). Interestingly, the increase in A-current density was inhibited by cycloheximide, a blocker of gene translation. Therefore, it was concluded that the effect of TNF-α might be mediated by de novo synthesis of channel protein itself and/or modulating proteins associated with the channel activities.

Altogether, the TNF-α/TNF-R2/NF-κB pathway may recruit several downstream targets in order to produce neuroprotective effects (Mattson, 2005) and the present study identified KCa2.2 as a central and novel candidate.

CHAPTER 4

Inhibition of Calpain Prevents *N*-Methyl-d-aspartate-Induced Degeneration of the Nucleus Basalis and Associated Behavioral Dysfunction

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ABSTRACT

N-Methyl-d-aspartate (NMDA) receptor-mediated excitotoxicity is thought to underlie a variety of neurological disorders, and inhibition of either the NMDA receptor itself, or molecules of the intracellular cascade, may attenuate neurodegeneration in these diseases. Calpain, a calcium-dependent cysteine protease, has been identified as part of such an NMDA receptor-induced excitotoxic signaling pathway. The present study addressed the question of whether inhibition of calpain can prevent neuronal cell death and associated behavioral deficits in a disease-relevant animal model, which is based on excitotoxic lesions of the cholinergic nucleus basalis magnocellularis of Meynert. Excitotoxic lesions of the nucleus basalis with NMDA induced a markedly impaired performance in the novel object recognition test. Treatment with the calpain inhibitor (A-705253), dose-dependently prevented the behavioral deficit. Subsequent analysis of choline acetyltransferase in the cortical mantle of the lesioned animals revealed that application of A-705253 dose-dependently and significantly attenuated cholinergic neurodegeneration. Calpain inhibition also significantly diminished the accompanying gliosis, as determined by immunohistochemical analysis of microglia activation. Finally, inhibition of calpain by A-705253 and the peptidic calpain inhibitor *N*-acetyl-Leu-Leu-Nle-CHO did not impair long-term potentiation in hippocampal slices, indicating that calpain inhibition interrupts NMDA excitotoxicity pathways without interfering with NMDA receptor-mediated signaling involved in cognition. We conclude that inhibition of calpains may represent a valuable strategy for the prevention of excitotoxicity-induced neuronal decline without interfering with the physiological neuronal functions associated with learning and memory processes.

4.1 INTRODUCTION

Calpains in the central nervous system are Ca^{2+}-dependent cysteine proteases, which can modulate the conformation, localization, and activity of various proteins. Calpain-induced modifications have a profound affect on biological processes as different as cell cycle progression and vesicular trafficking. They are an integrative part of several signaling cascades, but they can also contribute to apoptotic and necrotic cell death in pathological conditions. For this reason, calpains have been discussed extensively as target for therapeutic interventions in a number of neurodegenerative disease that are associated with neuronal loss (for review, see Huang and Wang, 2001; Goll et al., 2003; Zatz and Starling, 2005).

In that respect, it has recently been shown that inhibition of calpains prevents excitotoxic neuronal cell death in vitro (Caba et al., 2002; Ray et al., 2006) and in vivo (Chiu et al., 2005; Takano et al., 2005). Excitotoxicity is caused by an overstimulation of *N*-methyl-d-aspartate (NMDA) receptors, and there is now growing evidence that calpain cleaves several downstream targets that are critical for the progression of excitotoxic neuronal degenerative processes (Wu et al., 2004; Hou et al., 2006).

We have recently introduced a highly specific low-molecular-weight inhibitor of calpain, A-705253, which does not affect the multifunctional proteasome complex (Lubisch et al., 2003). Selectivity against the proteasome is necessary to evaluate the role of calpain, because the ubiquitin-proteasome system also contributes to some neurodegenerative diseases (Rubinsztein, 2006; Pan et al., 2008). By using such a specific calpain inhibitor, we addressed the questions of whether inhibition of calpain-mediated cascades would 1) prevent neurodegeneration in a model relevant for neurodegenerative diseases, 2) maintain the behavioral function of the affected brain area, and 3) affect normal NMDA receptor-dependent physiology. To analyze the neuroprotective effect of calpain inhibition, we used an established model of excitotoxicity-induced nucleus basalis magnocellularis (NBM) degeneration in rats (Horváth et al., 2000; Harkany et al., 2001, 2002). The NBM in rats is analogous to the nucleus basalis of Meynert in humans. Cholinergic fibers originating from the NBM extensively project to the neocortical mantle and play an essential role in the attention phase of information storage processing (Blokland 1995; Van der Zee and Luiten 1999). Lesioning of the NBM in rodents causes a degeneration of cholinergic cortical projections, which inevitably leads to severe cognitive deficits in affected rats and mimics the characteristic loss of forebrain cholinergic innervation in Alzheimer's disease (Bartus et al., 1982; Gaykema et al., 1992). Compounds that interfere with downstream calcium-associated intracellular pathways initiated by glutamate-induced calcium influx may well affect signaling cascades that are crucial for memory processes. To assess whether calpain inhibition would affect normal NMDA-dependent physiology, we also examined long-term potentiation (LTP) in hippocampal slices.

In this study, we present data showing that calpain inhibition prevents NMDA-induced neurodegeneration of NBM and associated decrease of cortical cholinergic innervation. Furthermore, calpain inhibition attenuates cognitive deficits that occur as a result of such neurodegeneration. Normal NMDA receptor-dependent physiology is not affected, indicating that only those downstream cascades are interrupted, which contribute to cellular pathology.

4.2 MATERIAL AND METHODS

4.2.1 Materials

A-705253 (Lubisch et al., 2003) was solubilized in 0.9% sodium chloride with a pH between 5 and 5.5, and injected intraperitoneally in doses of 1, 3, and 10 mg/kg b.wt. NMDA (Sigma-Aldrich, St. Louis, MO) solution was prepared in phosphate-buffered saline (PBS) solution (pH7.4) in a concentration of 60 nmol per 1 µl. The selective noncompetitive NMDA receptor antagonist (+)-MK-801 hydrogen maleate (Sigma-Aldrich) was dissolved in 0.9% sodium chloride for intraperitoneal injection in a dose of 2.5 mg/kg. *N*-Acetyl-Leu-Leu-Nle-CHO (ALLN; Calbiochem, San Diego, CA) stock solutions were prepared in dimethyl sulfoxide.

4.2.2 Animals and Surgical Procedure

Eight-month-old male Wistar rats (8–11 per group; Harlan, Borchen, Germany) were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg b.wt.). All care and treatments were carried out in accordance with the European Communities Council Directive on the use of experimental animals. One microliter of the freshly prepared solution of NMDA (60 nmol) in PBS was applied by a Hamilton microsyringe into the right nucleus basalis (anterior-posterior coordinate, -1.5 mm; lateral, 3.2 mm) at two dorsoventral positions, 6.2 and 7.0 mm ventral to the dura. Sham controls followed the same surgical procedures, but they were injected with the same quantity of the PBS alone. For more methodological details, we can refer to our earlier report (Luiten et al., 1995).

4.2.3 Drug Administration

A-705253, MK-801, or vehicle were applied 1 h before NMDA injection, which was followed by a second application 12 h after surgery and 2 daily injections for the next 2 days. MK-801 was also injected 1 h before and 12 h after surgery, but it was given only once per day for the two postsurgery days (at 2.5 mg/kg).

At postoperative day 12, the animals were transcardially perfused under deep pentobarbital anesthesia with a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, after a short prerinse with heparinized saline. The brains were removed, post-fixed for 2 days in the same fixative, and stored in phosphate buffer at 0°C, then cryoprotected by 30% sucrose for 4 days, and sectioned on a cryostat microtome at a thickness of 20 μm. Free-floating brain sections were processed for immunocytochemical staining.

4.2.4 ChAT Staining of Cholinergic Fibers

An immunostaining procedure was applied to visualize choline acetyltransferase (ChAT)-positive cholinergic neurons in NBM and their projection axon ramifications in the target brain areas, i.e., in the parietal neocortex. Every eighth section was stained for ChAT and CD11b as a microglia marker. Goat anti-ChAT primary antibody (Chemicon, Temecula, CA) was used in a 1:500 dilution. Biotinylated rabbit anti-goat antibody and Vectastain ACB kit was obtained from Vector Laboratories (Burlingame, CA). The staining was completed with nickel-enhanced diaminobenzidine reaction in the presence of H_2O_2.

4.2.5 Visualization of Microglial Activation by CD11b Antibody

Mouse anti-rat integrin αM [CD11b] monoclonal antibody (Chemicon) was used in a dilution rate of 1:1500 to recognize activated microglia. The biotinylated secondary antibody and the ABC kit were obtained from Vector Laboratories (see above). Diaminobenzidine reaction was enhanced by nickel ammonium sulfate, as described above.

4.2.6 Quantification of Cholinergic Fiber Density

The quantification procedure for cholinergic fiber density is established in our laboratory and was published several times in the past (Horváth et al., 2000; Harkany et al., 2001). In brief, the parietal neocortex, topographically corresponding to the anterior-posterior site of the NMDA lesion in the NBM and with the highest level of cholinergic fiber loss, was analyzed for fiber density with the Quantimet 600HR (Leica, Wetzlar, Germany) computer program. The exact measurement took place in the superficial sublayer of the layer V cortical area, which receives the highest density of cholinergic innervation. Three brain sections were analyzed per animal, and the results were averaged. Fiber density in percentage surface area of positively stained fibers was computed in both sides of the brain section. The ChAT-positive fiber density ipsilateral to the lesion was compared with the intact contralateral side, and the percentage loss was calculated as an indicator of cholinergic neuron and fiber degeneration.

4.2.7 Magnitude of Microglial Reaction

Microglial activation around the lesion was remarkably localized and could be quantified with a computerized technique that measured the size of the brain region infiltrated by the activated microglia (Quantimet; Leica). Area of microglia activation was manually delineated and measured at the level of NBM injection indicated by the overstained injection channel. Here again, three sections were selected from a total of 10 to 12 for measurements. The selection of sections and measurements were unbiased and carried out blindly.

4.2.8 Behavioral Testing

4.2.8.1 Small Open-Field Behavior

Seventy-two hours postsurgery, the behavior of the animals was assessed in a light-independent small open-field paradigm (Nyakas et al., 1991). Animals were placed into individual Perspex arenas, 24 × 24 cm in diameter with 30-cm high transparent walls, for 25 min. Six rats were observed simultaneously, and every 10 s the following behaviors were scored by a sampling technique: 1) rearing; 2) sniffing with head turning; 3) walking; 4) grooming; and 5) immobility (resting). Ambulation was also expressed by a combined score of rearing, sniffing, and walking (ambulation = 3 × rearing + 1 × sniffing + 2 × walking scores). The representative scores of each behavioral component were summed up in 5-min blocks and analyzed statistically.

4.2.8.2 Novel Object Recognition

Novel object recognition was assessed 5 days postsurgery. A day before testing novel object recognition ability, the rats were placed into a cylindrical open-field arena of 80-cm diameter surrounded by a 60-cm high reflective aluminium wall for 3 min. During this period, the rats were allowed to habituate to the experimental surroundings. The next day, during the 1st session of the novel object recognition test, the rats were allowed to explore the same open-field apparatus for 5 min while two identical objects (A + A) were placed into the arena. During the 2nd session, carried out 3 h after the 1st session, one of the two objects was replaced by a novel one (B + A). The ratio of visiting the novel versus known objects illustrated the object recognition ability, i.e., the attention behavior. The total period spent with exploration of the objects during the 1st and 2nd sessions were recorded. The recognition ability of the novel object at the 2nd session was calculated in the following way: duration of exploration of the novel (B) object was divided by the duration of exploration of both novel (B) and familiar (A) objects and expressed in percentages.

4.2.9 Electrophysiology

Seven to 8-week-old male Wistar rats (Harlan) were decapitated, and 400-µm transverse hippocampal slices were prepared with a self-constructed tissue chopper. The slices were transferred to a submerged recording chamber and adapted for at least 1 h at 33°C in artificial cerebrospinal fluid (124 mM NaCl, 4.9 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM CaCl, 1.2 mM KH_2PO_4, 25.6 mM $NaHCO_3$, and 10 mM glucose 10, pH7.4).

The Schaffer collateral was locally stimulated by a monopolar stimulation electrode (impedance 20 - 150 Ω; Sprint Metall Work, Reichshof, Germany) using a constant current, biphasic stimulus generator (voltage, 1–5 V; impulse duration, one polarity 0.1 ms and whole impulse 0.2 ms) (2100; AM-Systems Isolated Pulse Stimulator; AM-Systems, Carlsborg, WA). Field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum with glass electrodes (borosilicate glass with filament, 1–5 MOhm, diameter: 1.5 mm, tip diameter: 3–20 µm) filled with experimental solution. The field potentials were filtered by a low-pass filter (5 kHz). For the statistical analysis of the experiments, the slope of the fEPSP was determined. Recording, analysis, and supervision of the experiments were performed by means of a software program (PWIN), which was developed at the Leibniz Institute for Neurobiology (Department of Neurophysiology) in Magdeburg, Germany. LTP was induced using a theta burst stimulation protocol (four times two paired pulses were applied in intervals of 200 ms, the interval between the paired pulses was 10 ms, and the width of a single pulse was 0.2 ms).

4.2.10 Effect of A-705253 on Spectrin Cleavage in Hippocampal Slice Cultures

Hippocampal slices were prepared from 9 to 11-day-old Wistar rats. Slices, 350-µm thick, were placed on Millicell single-well PCF inserts (Millipore, Schwalbach, Germany) in 6-well cell culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and kept in an incubator for 8 days at 34°C with 5% CO_2 air. The slices were cultured in medium A [25 mM HEPES, pH 7.3, 40% basal medium Eagle with Earle's salt, 25% horse serum, 25% Earle's balanced salt solution, 1 mM GlutaMAX (Invitrogen GmbH, Karlsruhe, Germany), and 28 mM glucose] for the first 3 days. Thereafter, medium A was replaced with Neurobasal A medium (Neurobasal A medium, 1 mM GlutaMAX, and 25 mM glucose; Invitrogen GmbH), and the slices were kept in culture for an additional 5 days. Neurobasal A medium was replaced twice a week.

After cultivation of the hippocampal slices for 8 days, the medium was aspirated and replaced with 1 ml of Neurobasal A medium. A total of 0.001 ml of different A-705253 inhibitor solutions at 10, 1, and 0.1 mM was added, respectively. After a 30-min incubation at 34°C with 5% CO_2 air, NMDA was added (10 µM final

concentration) and incubated for 5 h at 34°C with 5% CO_2 air. After this time, the slices were collected in 1.5-ml Eppendorf tubes and solubilized by the addition of 0.025 ml of lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% sucrose, 0.1% CHAPS, 1 mM dithiothreitol, 1:25 CompletePlus (Roche Diagnostics GmbH, Mannheim, Germany), and 1:100 phosphatase inhibitor cocktail set II (Calbiochem-Merck Biosciences GmbH, Darmstadt, Germany). Slices were homogenized with a plastic pistill and kept on ice for 5 min. The insoluble material was removed by centrifugation for 5 min at 13,000 rpm and 4°C, and the supernatant was collected. Supernatant protein concentrations were determined with MicroBCA (Pierce Inc., Rockford, IL). Samples (equal total protein of 20 μg/lane) were mixed with NuPAGE LDS sample buffer 4× (Invitrogen GmbH), heated for 5 min at 95°C, and thereafter resolved by 4 to 12% SDS ± polyacrylamide gel electrophoresis (Invitrogen GmbH). After transfer of proteins to nitrocellulose membranes, the proteins were analyzed by immunoblotting using a murine monoclonal antibody (mAB 1622; Chemicon) specific for spectrin and calpain-mediated spectrin breakdown products (SBDPs) followed by a peroxidase-conjugated secondary sheep anti-mouse antibody (A6782; Sigma-Aldrich) and a peroxidase substrate. To determine the percentage inhibition, the integrated optical density of the SBDPs in the presence and absence of inhibitor was determined using the VersaDoc Imaging System (Bio-Rad Laboratories GmbH, München, Germany).

4.3 RESULTS

4.3.1 Calpain Inhibition Markedly Attenuates Degeneration of Cholinergic Fibers after Excitotoxic Lesion of the Nucleus Basalis Magnocellularis

We analyzed the effect of calpain inhibition on the cholinergic innervation of the parietal neocortex after induction of an excitotoxic insult to the nucleus basalis magnocellularis in rats. Immunostaining visualized ChAT-positive cholinergic innervation fibers in the parietal neocortex (Figure 4.1, 4.2), and fiber density was measured in the superficial sublayer of the layer V as described in Horvath et al. (2000). The ChAT-positive fiber density ipsilaterally to the lesion was compared with the intact contralateral side and the percentage of fiber loss in the various experimental groups summarized in Figure 4.3. Injection of NMDA to the NBM decreased the density of the ChAT-positive fibers in the parietal cortex by 50%. Application of 2.5 mg/kg MK-801 nearly completely prevented this decrease. The calpain inhibitor A-705253 dose-dependently protected the cholinergic neurons and their projection fibers against the excitotoxic injury and reduced the fiber degeneration with 44 and 49% with 3 and 10 mg/kg, respectively. Application of

10 mg/kg A-705253 did not affect the density of the cholinergic innervation, when given to sham-treated animals.

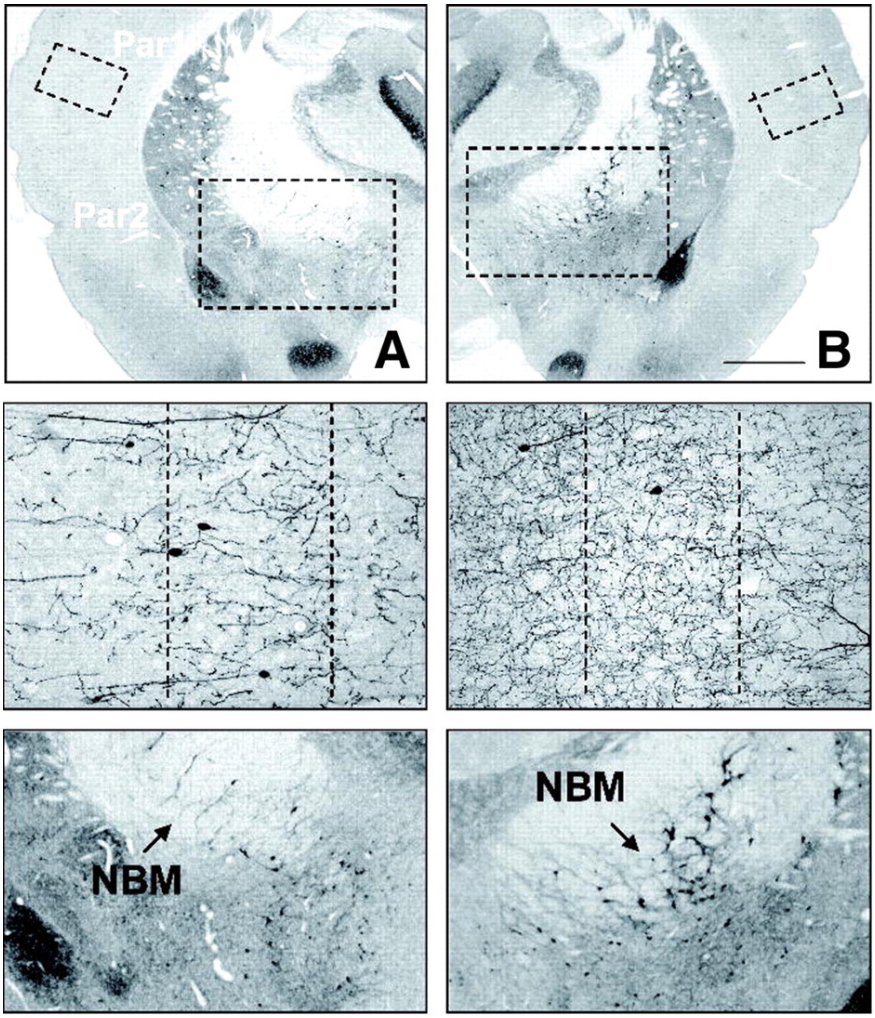

Figure 4.1. *Anatomical description of studying the cholinergic system after NBM lesion with NMDA. A, the posterior part of the NBM was lesioned, and most of the magnocellular cholinergic neurons are absent after lesion (lower left panel, which is the magnification of the nucleus basalis area outlined by dashed lines at A). The parietal neocortex of lesioned rat represents a marked loss of ChAT-positive fibers (middle left panel). The precise anatomical region between parietal cortex area 1 (Par1; primary somatosensory cortex) and parietal cortex area 2 (Par2; supplementary somatosensory cortex) (Zilles 1985), where most of the areas were sampled for quantitative measurements of fiber density, is outlined by dashed lines. B and the right panels represent the nonlesioned control side of the same rat. The bar in B is 1 mm.*

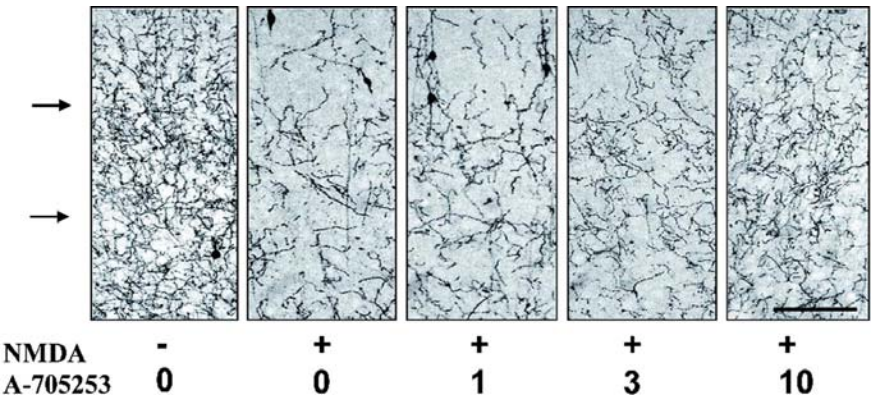

Figure 4.2. *Photomicrographs of cholinergic innervation of the parietal neocortex in 5 groups of animals: sham-operated and vehicle-treated, NMDA-treated and vehicle-injected, NMDA-treated and A-705253-injected in three different doses of 1, 3, and 10 mg/kg (labeled 1, 3, and 10, respectively). Quantitative measurement of fiber density was performed between the two arrows in the superficial part of layer V. The bar at the right represents 100 μm.*

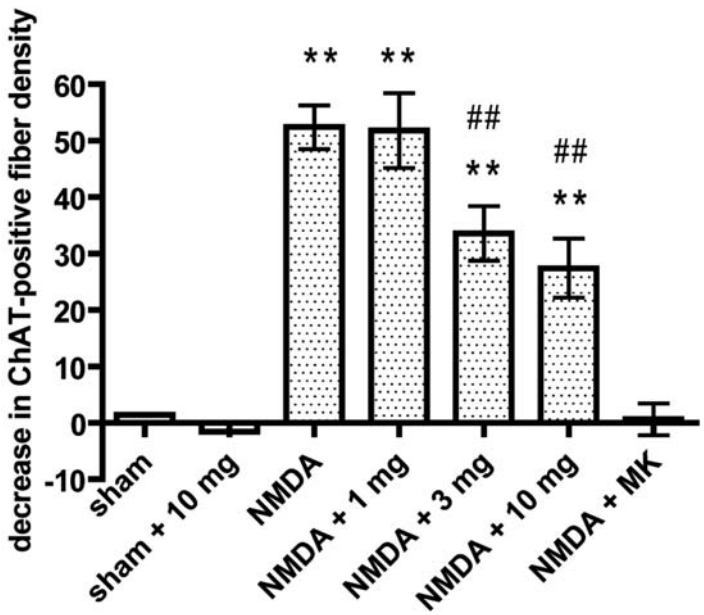

Figure 4.3. *Attenuation of cholinergic neurodegeneration by calpain inhibitor A-705253. Groups are sham-lesioned and NMDA-treated, and they received injections with either the vehicle or the different doses of A-705253 (1, 3, and 10 mg/kg) or MK-801 (MK). **, $p < 0.01$ versus sham-lesioned and saline-treated groups; not significant from sham-0; ##, $p < 0.01$ versus NMDA-injected and vehicle-treated groups.*

4.3.2 Calpain Inhibition Decreased Microglia Activation after Excitotoxic Lesion of the Nucleus Basalis Magnocellularis

We further sought to examine whether calpain inhibition would prevent microglial activation that normally accompanies neurodegenerative processes. Therefore, we measured the area of microglial activation at the level of the NBM injection by staining microglia for integrin. Injection of NMDA strongly increased this area, which corresponds to the spread of the neurotoxin in the injection locus (Harkany et al., 2000) (Figures. 4.4 and 4.5). When rats were treated with calpain inhibitor A-705253, the area of activation was dose-dependently decreased, and it reached significance at a dose of 3 mg/kg. The NMDA receptor antagonist MK-801 also strongly reduced the area size of microglia activation.

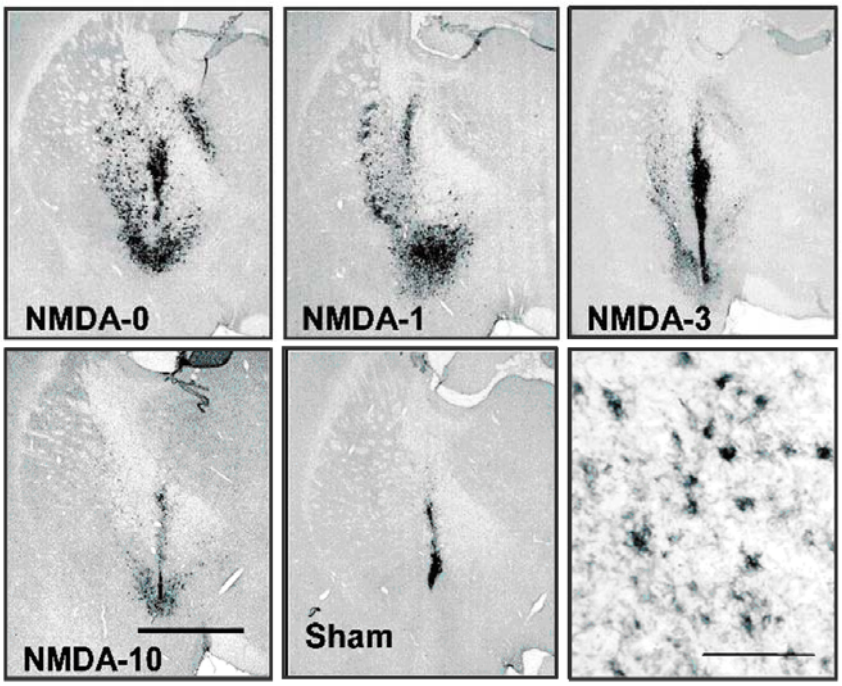

Figure 4.4. *Photomicrographs show the extent of activated microglia reaction at the level of brain injection indicated by the stained injection channel in representative rats of five different experimental groups: sham-operated and vehicle-treated (sham-0), NMDA-treated and vehicle-injected (NMDA-0), NMDA-treated and A-705253-injected in three different doses of 1, 3, and 10 mg/kg (NMDA-1, NMDA-3, and NMDA-10, respectively). For quantitative measurement of the extent of microglia activation, the total surface area was quantified that was infiltrated by activated microglia. In the right upper panel, individual activated microglia are shown to be immunoreactive for CD11b with higher magnification (bar represents 200 μm). The bar at the right lowest panel shows 1 mm.*

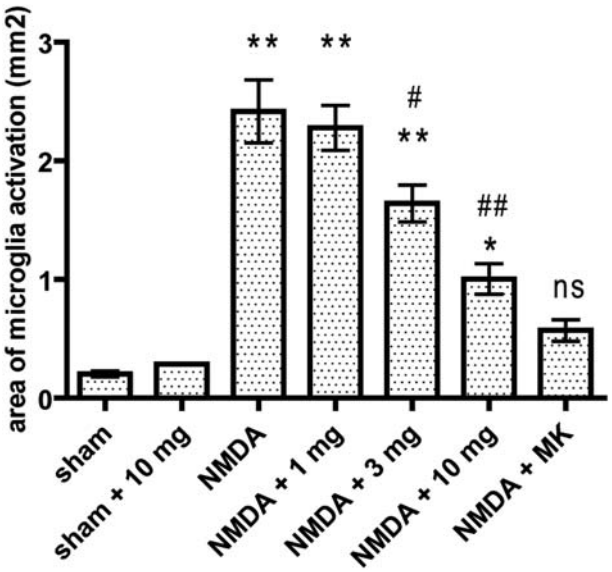

Figure 4.5. *Attenuation of microglia activation by calpain inhibitor A-705253. Groups are sham-lesioned and NMDA-treated, and they received injections with either the vehicle (0 mg) or the different doses of A-705253 (1, 3, and 10 mg/kg) or MK-801 (MK). *, p < 0.05 and **, p < 0.01 versus sham-lesioned and saline-treated group; ns, not significant from the sham group; #, p < 0.05 and ##, p < 0.01 versus NMDA-injected and vehicle-treated groups.*

4.3.3 Calpain Inhibition As Well As NMDA Lesioning Does Not Affect Behavior in the Small Open-Field Test

We further examined whether NMDA lesioning of the NBM or the drug treatment itself would influence the rats ability to perform spontaneous behavior in an open-field test (experiments were performed blindly). No significant effects of the NMDA lesion and of the treatment with A-705253 were recorded at day 4 after the lesion. The different components of ambulatory behavior, i.e., rearing, walking, and sniffing with head movements, were not different from sham-treated control levels (data are not shown). Time spent with grooming and immobility was also unaltered (Figure 4.6). Ambulation in general was not influenced by the different experimental manipulation. Therefore, the general condition to perform spontaneous behavior was not significantly altered by the lesion and by drug treatment.

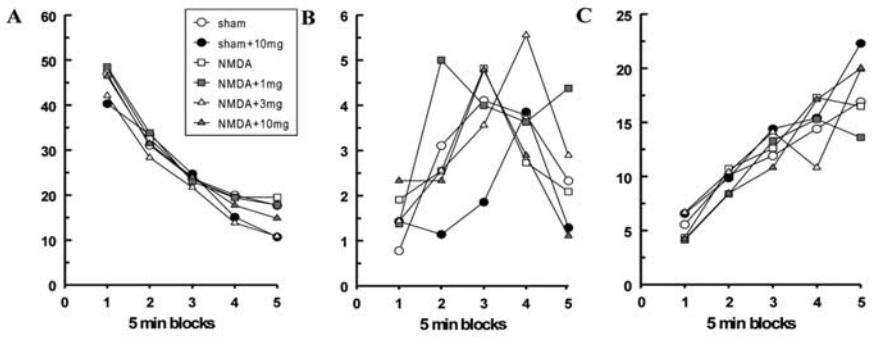

Figure 4.6. *Components of behavioral activity in small open field are shown. Ambulation expressed as a combined score of rearing, walking, and sniffing is depicted in A, grooming is depicted in B, and immobility is depicted in C. Scores collected by behavioral sampling every 10 s for 25 min are shown in blocks of 5 min. No statistically significant difference could be obtained between groups.*

4.3.4 Calpain Inhibition Attenuated Cognitive Deficits Occurring after Excitotoxic Lesion of the Nucleus Basalis Magnocellularis

After habituation to the open-field box, attention was tested at day 6 (5 days after surgery; experiments were performed blindly). The results obtained are shown in Figure 4.7A. Spontaneous exploration of different objects placed in the open-field arena was not influenced by the lesion or by the treatments. MK-801-treated rats explored somewhat less, but this change was not significant. The objects were placed on the floor of the open field, and thus they could be approached by horizontal ambulation. This was not different among the groups.

Recognizing the novel object in contrast to the familiar one was markedly decreased by the NMDA lesion (Figure 4.7B). Treatment with A-705253 dose-dependently prevented the deficit. ANOVA revealed a significant difference between sham control and lesioned and A-705253-treated groups ($p < 0.005$). The smallest dose of A-705253 was ineffective. The dose of 3 mg/kg attenuated the effect of the NMDA lesion, but the interference just approached statistical significance ($p = 0.074$). However, performance of this group was closer to the values of sham controls ($p = 0.104$). The highest dose of 10 mg/kg completely prevented the effect of lesion ($p < 0.005$). The NMDA-lesioned group that was treated with MK-801 also displayed an attention level to novel stimuli comparable with the sham-lesioned controls.

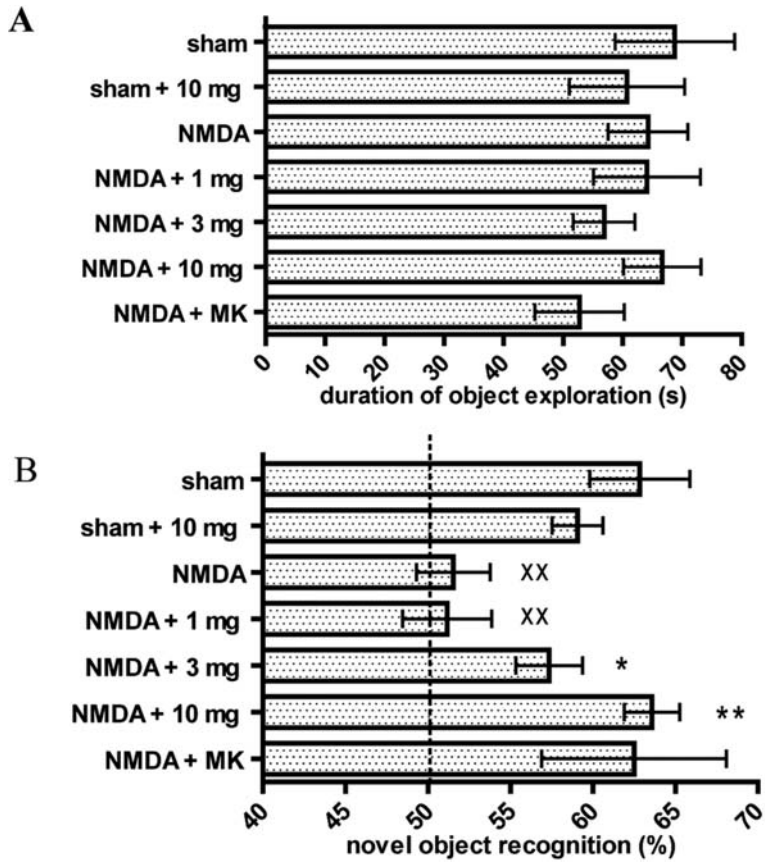

Figure 4.7. *Exploratory behavior in the novel object recognition test. A, the duration of exploration of objects irrespective of their nature; B, the relative preference of novel object exploration compared with the familiar object. The different treatments did not influence object exploration in general. The NMDA lesion decreased attention toward the novel object, and A-705253 treatment interfered with the lesion effect. The dotted line at 50% represents the chance level. xx,* $p < 0.01$ *versus sham-operated control; *,* $p < 0.05$ *and **,* $p < 0.01$ *versus NMDA-lesioned group.*

4.3.5 Calpain Inhibition Did Not Affect NMDA Receptor-Dependent LTP

To examine whether general NMDA receptor-mediated physiological functions and related intracellular signaling cascades were affected by calpain inhibition, we analyzed LTP during administration of A-705253 in hippocampal slices (Figures 4.8, A and C). Calpain inhibition by A-705253 did not affect LTP at 100 nM, which is comparable with brain levels of A-705253 at the dose of 10 mg/kg. We also tested the peptidic inhibitor ALLN for possible effects on LTP (Figure 4.9, A

and B), but we did not detect any LTP suppression. Basic synaptic transmission was also unaltered, as assessed by the input/output relation (Figures 4.8B and 4.9C). To determine whether the LTP protocol used in this study was indeed NMDA receptor-dependent, we used the same LTP induction paradigm during treatment with the NMDA receptor blocker APV (Figure 4.9D). LTP was completely blocked under this condition, indicating that the LTP induction with our tetanization paradigms in CA1 was indeed NMDA receptor-dependent.

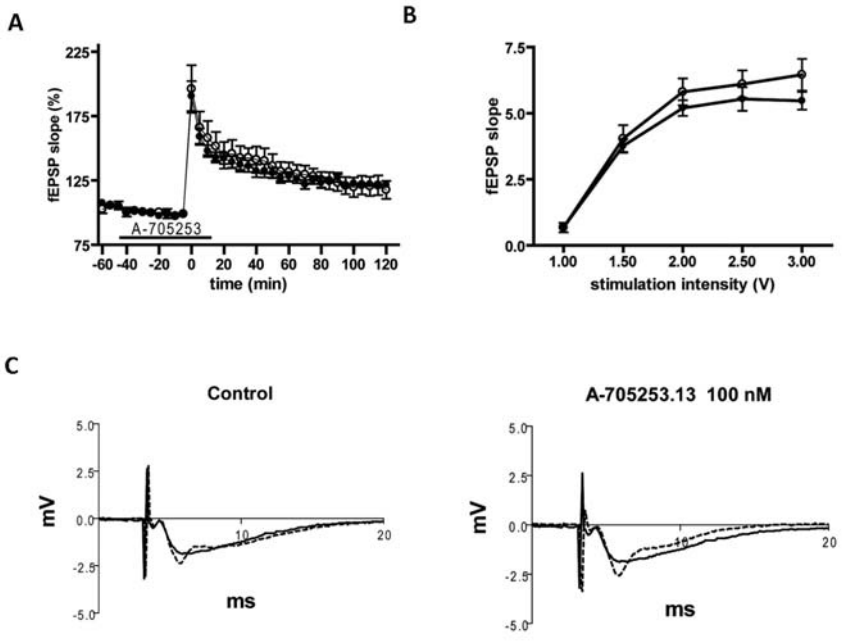

Figure 4.8. *Effect of A-705253.13 (100 nM) on the potentiation of the fEPSPs after a weak tetanus. A, averaged LTP time course. When applied 45 min before until 10 min after a weak tetanus, A-705253.13 (100 nM) did not affect LTP elicited by weak tetanization (control:* n = *10 slices, 5 animals; A-705253.13:* n = *10 slices, 5 animals;* p = *0.742, repeated measures ANOVA). B, input/output relation. C, original traces taken before (solid line) and after the tetanus (dotted line). Open circles, control; filled circles, 100 nM A-705253.*

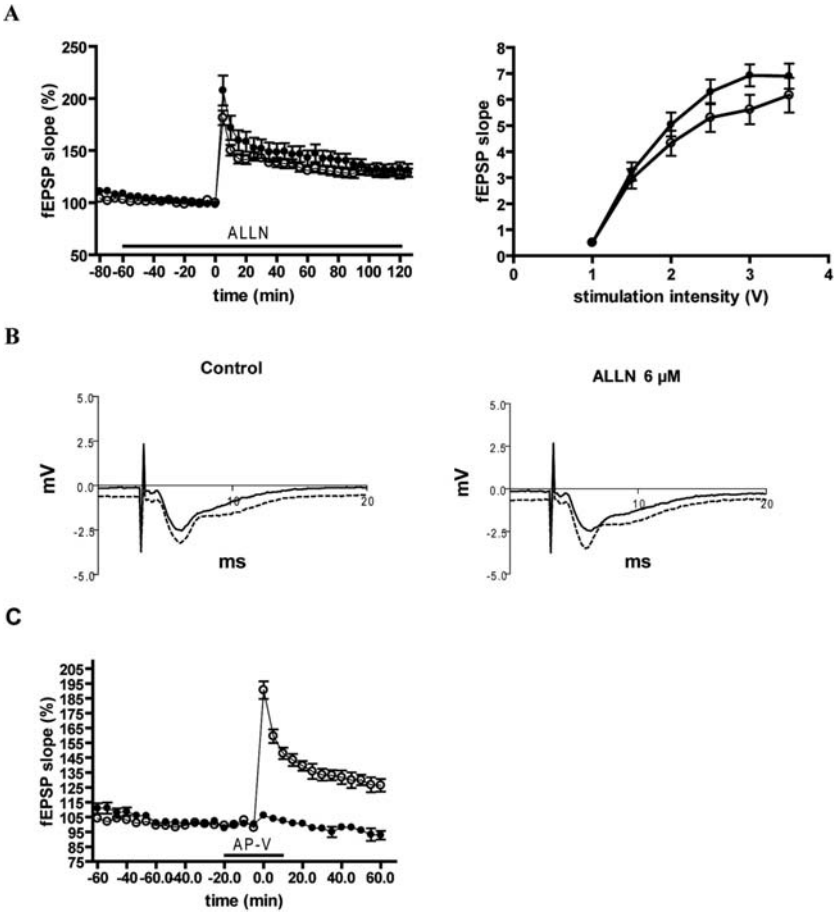

Figure 4.9. *Effect ALLN and AP-V on the potentiation of the fEPSPs after a weak tetanus. A, averaged LTP time course under ALLN (left panel). When ALLN (6 µM) was applied 60 min before until 120 min after tetanization (6 µM), LTP was not affected (dimethyl sulfoxide control:* n *= 10 slices, 5 animals; ALLN:* n *= 10 slices, 5 animals;* p *= 0.230, repeated measures ANOVA). Right panel, input/output-relation. B, original traces taken before (solid line) and after the tetanus (dotted line). C, averaged LTP time course under APV. When APV (AP-V) (25 µM) was applied 20 min before until 10 min after tetanization, LTP was completely suppressed (control:* n *= 5 slices, 3 animals; APV:* n *= 5 slices, 3 animals). Open circles, control; filled circles, compound.*

4.3.6 Calpain Was Activated by NMDA in Hippocampal Slice Cultures and Dose-Dependently Inhibited by A-705253

To examine whether application of NMDA activates calpain in neurons, and whether A-705253 sufficiently inhibits the activated calpain, we cultured rat hippocampal slices and exposed them to NMDA in the presence or absence of

different concentrations of A-705253. Spectrin was cleaved under 10 μM NMDA treatment into its spectrin degradation products of 150/145 kDa (Figure 4.10A), indicating that calpain was indeed activated in neurons treated with NMDA. Application of A-705253 dose-dependently prevented calpain activation, as indicated by a decrease of SBDPs. The calculated IC_{50} is 408 nM = 29 nM for free calpain inhibitor (considering 93% protein binding for A-705253; Figure 4.10B).

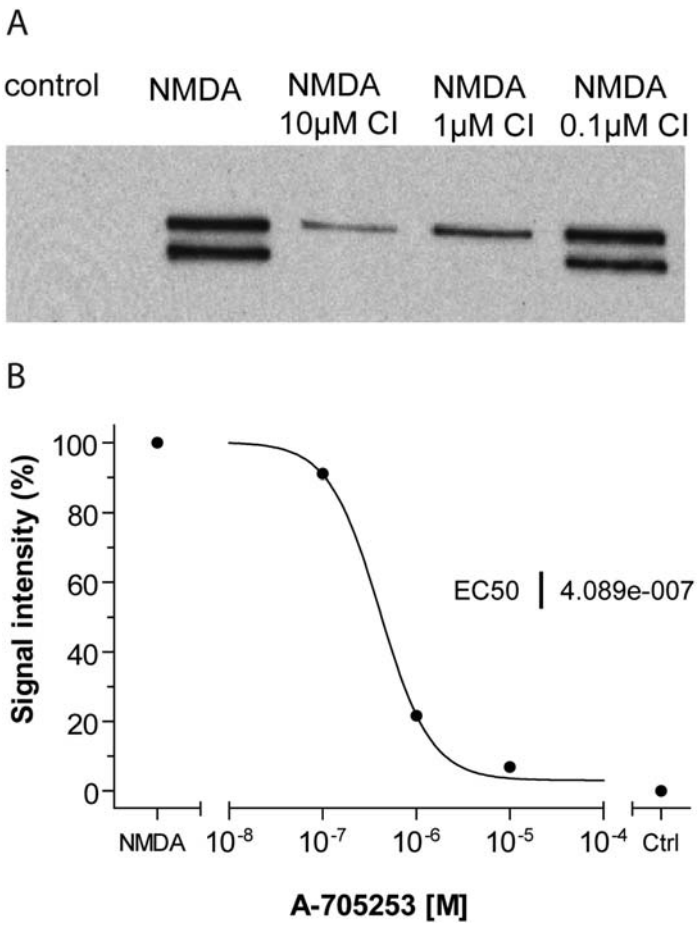

Figure 4.10. *Spectrin cleavage in rat hippocampal slice cultures for the determination of calpain activity. A, immunoblot of slice cultures treated with 10 μM NMDA in the absence and presence of calpain inhibitor A-705253 (CI) using an antibody directed against spectrin and spectrin degradation products. The 150/145-kDa spectrin degradation bands are shown in the image. Control lanes are untreated cultures. B, IC_{50} for calpain inhibition by A-705253 determined by graphical analysis of the 150/145-kDa band signal intensities at different inhibitor concentrations. The NMDA band was used as 100%.*

4.4 DISCUSSION

Excitotoxic cell death has been discussed as a common principle for several progressive neurological disorders (Lipton and Rosenberg, 1994; Harkany et al., 2000; Mattson, 2004). Inadequate control over NMDA receptor stimulation causes excessive calcium influx into neurons, activating a subset of intracellular Ca^{2+}-dependent signaling cascades. As a result, affected cells perish, can eventually die, and consequently the functionality of affected brain areas is impaired. During this process, Ca^{2+}-activated calpain cleaves several proteins that are involved in cell death signaling. For example, Wu et al. (2004) provide data that cleavage of calcineurin by calpain contributes to neuronal degeneration. Xu et al. (2007) could show that truncation of mGluR1α by calpain is essential to excitotoxicity. Furthermore, collapsin response mediator-protein 3 triggers neuronal death after cleavage by calpain (Hou et al., 2006). In a more chronic model, Kim et al. (2007) could show that degeneration of cortical neurons in vitro depends on calpain cleavage of p35. If calpain is part of such degenerative cascades, then inhibition of calpain should attenuate neuronal decline. Indeed, Caba et al. (2002) could show that inhibition of calpain was neuroprotective in hippocampal slice cultures exposed to NMDA receptor overstimulation. In vivo, Chiu et al. (2005) revealed that calpain inhibition should reduce neurodegeneration in the rat retina. For these reasons, calpain has been discussed as a target for neurodegenerative disorders (Huang and Wang, 2001; Zatz and Starling, 2005; Saez et al., 2006).

The data presented here strongly suggest calpain that inhibition by A-705253 is neuroprotective in a model of NMDA-induced cholinergic cell lesion. After such cholinergic basal forebrain damage by NMDA and subsequent cholinergic cortical denervation, learning behavior was compromised as assessed in a novel object recognition test, in which a dose-dependent protective effect of A-705253 on behavioral decline was demonstrated. Anatomical findings further confirmed the neuroprotective effect of calpain inhibition. Both the degree of cholinergic denervation in the parietal cortex and the extent of microglia activation around the injection in the NBM were markedly and dose-dependently attenuated by the inhibitor. The effective dose was shown to inhibit calpain in vivo, as determined by experiments on tau phosphorylation in the rat brain (data not shown). The present findings support the conclusion that inhibition of calpain not only prevents neuronal decline in an animal model of excitotoxicity, but it also attenuates accompanying behavioral deficits and neuropathology, including gliosis.

Most of the previous studies on calpain inhibition used peptidic inhibitors that are not specific for calpain. Furthermore, due to low solubility, these compounds had to be applied at high concentrations, which again may have resulted in unspecific side effects. The compound used in the present study was recently introduced by Lubisch et al. (2003), and it has a high inhibitory potency for calpain (K_i = 27 nM) without affecting the proteasome (K_i > 10,000 nM) and caspases (K_i > 10,000 nM).

Therefore, the observed neuroprotective effects are probably mediated by inhibition of calpain and not by reducing proteasome or caspase activity. This finding is important in light of the fact that the proteasome complex contributes to a variety of neurodegenerative disorders (Rubinsztein, 2006; Pan et al., 2008). A-705253 has previously been shown to rapidly and effectively penetrate myocardial tissues, and it exerts its effects on cellular targets within minutes after perfusion (Neuhof et al., 2003, 2004), making it an ideal compound for studying calpain in vivo.

Excitotoxicity-induced neurodegeneration is caused by an overactivation of the NMDA receptor (Liu et al., 2007; von Engelhardt et al., 2007). Consequently, blockade of the NMDA receptor has been widely discussed and tested as a pharmacological target for excitotoxicity-induced neurodegeneration (Harkany et al., 1999; Lipton 2004; Gardoni and Di Luca, 2006). However, one of the caveats of this approach is that a functional NMDA receptor is indispensable for a variety of physiological and behavioral brain functions, including synaptic plasticity, long-term potentiation, and memory performance (Lynch, 2004; Fedulov et al., 2007), and it is well established that blockade of the NMDA receptor impairs cognition (Morris, 1989; Fedulov et al., 2007). In the study presented here, calpain inhibition by A-705253 was similarly effective as MK-801 against the excitotoxic insult. This indicates that blocking the calpain-mediated downstream pathway in specific neurotoxic conditions can be as effective in suppressing the NMDA receptor. Therefore, we investigated whether calpain inhibition would also prevent or affect normal physiological function of the NMDA receptor, as does MK-801. To address this question, we examined LTP in the hippocampal CA1 region under exposure to A-705253. LTP at the Schaffer collateral requires functional NMDA receptors (Harris et al., 1984; Reymann et al., 1989). We did not detect any LTP impairment during calpain inhibition, allowing the conclusion that normal NMDA receptor function and downstream calcium-dependent mechanisms of synaptic plasticity are not affected by A-705253 treatment. The concentration used effectively inhibited calpain in hippocampal slices as well as cultured neurons, as determined by tau and dynamin degradation as well as tau hyperphosphorylation (data not shown). LTP during application of the peptidic calpain inhibitor ALLN also yielded LTP equal to controls. To ensure that our LTP protocol indeed induces NMDA receptor-dependent LTP, the NMDA blocker (2*R*)-amino-5-phosphonovaleric acid (APV) was applied under the same protocol. Under this condition, LTP was completely prevented. Therefore, it is unlikely that calpain is involved with all of the cascades induced by NMDA receptor activation, and it may thus be a more appropriate target for neuroprotection than the NMDA receptor itself.

With the exception of traumatic brain injury and stroke, most neurodegenerative diseases progress slowly over a period of many years. The excitotoxic insult used in this model induced a more rapid decline, and behavioral deficits were already measurable after days. At this point, it is not known whether calpain inhibition would also prevent a more chronic neurodegeneration of the nucleus basalis, as

observed in Alzheimer's disease. Further experiments are needed to evaluate whether calpain inhibition can also counteract a chronic loss of cholinergic cells.

Taken together, this study shows that inhibition of calpain 1) is sufficient to prevent NMDA-induced degeneration of NBM and its associated cholinergic fiber projections, 2) attenuates behavioral deficits associated with such lesion, and 3) does not interfere with the physiological role of the synaptic NMDA receptor. These findings indicate that inhibiting calpain could be an attractive neuroprotective strategy, without interfering with physiologically relevant NMDA receptor-related processes and its behavioral functions.

CHAPTER 5

Calpain inhibition prevents amyloid-β-induced neurodegeneration and associated behavioral dysfunction in rats

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Neuropharmacology (submitted)

ABSTRACT

Amyloid-β (Aβ) is toxic to neurons and such toxicity is – at least in part – mediated via the NMDA receptor. Calpain, a calcium dependent cystein protease, is part of the NMDA receptor-induced neurodegeneration pathway, and we previously reported that inhibition of calpain prevents excitotoxic lesions of the cholinergic nucleus basalis magnocellularis of Meynert. The present study reveals that inhibition of calpain is also neuroprotective in an in vivo model of Aβ oligomer-induced neurodegeneration in rats. Aβ-induced lesions of the nucleus basalis induced a significant decrease in the number of cholinergic neurons and their projecting fibers, as determined by analysis of choline acetyltransferase in the nucleus basalis magnocellularis and cortical mantle of the lesioned animals. Treatment with the calpain inhibitor A-705253 significantly attenuated cholinergic neurodegeneration in a dose-dependent manner. Calpain inhibition also significantly diminished the accompanying neuroinflammatory response, as determined by immunohistochemical analysis of microglia activation. Administration of β-amyloid markedly impaired performance in the novel object recognition test. Treatment with the calpain inhibitor, A-705253, dose-dependently prevented this behavioral deficit.

In order to determine whether pre-treatment with the calpain inhibitor is necessary to exhibit its full protective effect on neurons we induced Aβ toxicity in primary neuronal cultures and administered A-705253 at various time points before and after Aβ oligomer application. Although the protective effect was higher when A-705253 was applied before induction of Aβ toxicity, calpain inhibition was still beneficial when applied up to 1 hour post-treatment.

We conclude that inhibition of calpains may represent a valuable strategy for the prevention of Aβ oligomer-induced neuronal decline and associated cognitive deterioration.

5.1 INTRODUCTION

According to the amyloid-β-hypothesis of Alzheimer's disease (AD) accumulation of Aβ in brain parenchyma – possibly in its soluble form – causes a degeneration of neurons and their processes in brain areas involved in memory formation (Selkoe *et al.,* 2008). Among the first regions to be affected are the hippocampus and the nucleus basalis of Meynert. The latter provides the majority of cholinergic input to neocortical structures and plays an essential role in attention and information storage (Blokland 1995; Van der Zee and Luiten 1999). Damage and the selective degeneration of the nucleus basalis of Meynert provides the morphological correlate of the cortical cholinergic deficiency in AD. The loss of this discrete cholinergic neuronal population leads to an impairment of higher cortical functions, which is directly related to the progressive deterioration of memory and attention, and cognitive processes in affected patients.

A number of studies suggest that Aβ-induced toxicity in AD is caused by excessive glutamate stimulation, over activation of the NMDA receptor, and subsequent calcium accumulation in the postsynaptic neuron (Harkany *et al.*, 2000; Molnar *et al.*, 2004; Mattson *et al.,* 2000). Recently, the pathology of Aβ has been correlated to oligomeric forms of the peptide (for review see Walsh and Selkoe, 2007), and studies indicate an involvement of the NMDA receptor also in oligomer toxicity (Shankar *et al.*, 2007). Although the exact mechanism of this process is not fully understood, there is evidence that calpains, Ca^{2+}-dependent cystein proteases, are components of the downstream cascade. Inhibition of calpains prevents excitotoxic neuronal cell death *in vitro* (Caba *et al.*, 2002; Ray *et al.*, 2006) and *in vivo* (Chiu *et al.*, 2005; Takano *et al.*, 2005), and there is evidence that calpain cleaves several downstream targets that are critical for the progression of excitotoxic neurodegeneration (Hou et al., 2006; Wu et al., 2004). Calpains have therefore been discussed as a target for interference in the neurodegenerative diseases that are associated with neuronal loss (for review see Huang and Wang, 2001; Goll *et al.*, 2003, Zatz and Starling, 2005).
Using a specific low molecular weight inhibitor, A-705253 (Lubish *et al.* 2003), we have recently shown that inhibition of calpain completely prevents NMDA-induced excitotoxic lesions of the nucleus basalis magnocellularis (NBM), the rat analog of the nucleus basalis of Meynert in humans. A-705253 also fully protected from behavioral deficits that accompany such lesions (Nimmrich *et al.*, 2008). Although excitotoxicity is likely to contribute to the pathology of AD, this study did not reveal whether neuronal decline could also be prevented, if the insult was induced by Aβ. To provide this missing link we assessed whether calpain inhibition would protect from Aβ-induced degeneration of the NBM, and whether such treatment would protect from associated cognitive decline of the rats.
As oligomeric Aβ is now thought to underlie the pathology of the disease, we generated Aβ-oligomers *in vitro* and used such oligomer preparation - rather than the monomeric peptide - to induce NBM degeneration in rats. Lesioning of the

NBM causes a decline of cholinergic projections, mimicking the characteristic loss of forebrain cholinergic innervation in AD (Bartus *et al.*, 1982; Gaykema *et al.*, 1992).
Here we present data showing that calpain inhibition prevents Aβ oligomer-induced neurodegeneration of NBM and associated decrease of cortical cholinergic innervation. Furthermore, calpain inhibition attenuates cognitive deficits that occur as a result of such neurodegeneration.
NMDA receptor activation is an early step in the excitotoxicity cascade, and compounds targeting the NMDA receptor have to be administered in close time proximity of the toxic stimulus. Calpain activation lies further downstream in this cascade, thus offering an opportunity to interfere with cell death signalling at later time points. We therefore added to this study an *in vitro* analysis of the time course of the calpain application relative to the point of insult. Calpain inhibition is also neuroprotective when initiation of the toxic insult has already been initiated.

5.2 MATERIAL AND METHODS

5.2.1 Calpain inhibitor

Calpain inhibitor *A-705253* was solubilized in DMSO (Sigma-Aldrich, St. Louis, USA) and stored as 1 M stock at -20 °C. Working stock solutions with different concentrations of A-705253 were prepared in ultrapure water containing 0.9% sodium chloride with a pH between 5 and 5.5. The solution was prepared freshly before use.

5.2.2 Preparation of Aβ-oligomers

Oligomeric $A\beta_{42}$ was prepared as was described by Dahlgren *et al.* (2002). In short, solid $A\beta_{42}$ peptide (EZBiolabs, Carmel, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)(Sigma-Aldrich, St. Louis, USA) to a concentration of 1 mM. The peptide solution was aliquoted and the HFIP removed by evaporation in a SpeedVac (Savant Instruments, Hyderabad, India). The dry peptide films were stored at -20 °C until further processing. Before use $A\beta_{42}$ films were dissolved in anhydrous DMSO to 5 mM and subsequently diluted in neurobasal medium to a final concentration of 100 μM (stock solution). The stock solution was incubated at 4 °C for 24 h to enable $A\beta_{42}$ oligomerization.

5.2.3 Animals

All animals were purchased from Harlan (Horst, The Netherlands). For the *in vivo* experiments, we used male Wistar rats of 3.5 months of age. For the *in vitro* experiments we used female C57BL/6J mice (12 weeks old). During the experiment

animals were kept under normal laboratory conditions in an air-conditioned room (21 ± 2°C) with a 12/12h light dark cycle (lights on at 07.00 h) with food and tap water ad libitum. All care and treatments were carried out in accordance with the European Communities Council Directive on the use of experimental animals.

5.2.4 Nucleus basalis lesion

Surgery was performed as described in Luiten et al. (1995). The animals were anaesthetized with Nembutal (sodiumpentobarbital, 60 mg/kg i.p.). The coordinates for the injection in the nucleus basalis magnocellularis (NBM) were 1.5 mm posterior to bregma, 3.2 mm lateral to midline as defined by the atlas of Paxinos and Watson (1986). A 5 µL Hamilton syringe was lowered into the brain, followed over 10 min by two injections of 0.5 µL of the freshly prepared solution of $A\beta_{1\text{-}42}$ oligomers (250 pmol each) diluted in 0.01 mM phosphate buffer pH 7.4 unilatterally at two dorsoventral positions, 6.0 mm and 6.7 mm ventral to the dura. The final injected amount of the peptide therefore was amounted to 500 pmol per animal. For sham-operated animals two times 0.5 µl phosphate buffered physiological saline solution were infused (0.01 mM pH 7.4 PBS) containing equivalent amount of DMSO, which served for sham-injection. After each injection the needle was left *in situ* for another 10 min to allow for diffusion and to limit spread of the solution during withdrawal of the needle. Brain injections were performed only in the right hemisphere and the left hemisphere was left undisturbed and served for the self-control side for the histological examinations.
The animals received the calpain inhibitor A-705253 intraperitoneally in doses of 1, 3, and 10 mg/kg of body weight, one hour before, 12 hours after and twice a day for two consecutive days after surgery (for experimental design see Figure 5.1).

5.2.5 Small open-field behavior

A moderate novelty-induced behavioral activation and habituation to a dimly lit home-cage like novel environment was tested in this paradigm (Nimmrich et al., 2008). The test also reflected the general behavioral condition after experimental manipulations, since it was performed 3 days after the surgery. Every 10 seconds the following behaviors were scored by behavioral sampling technique: a) rearing, b) sniffing with head turning, c) walking, d) grooming, and e) immobility (resting). Exploration was expressed by a combined score of rearing, sniffing and walking (exploration = 3 x rearing + 1 x sniffing + 2 x walking scores). The representative scores of each behavioral component were summed up in 5 min blocks and analyzed statistically.

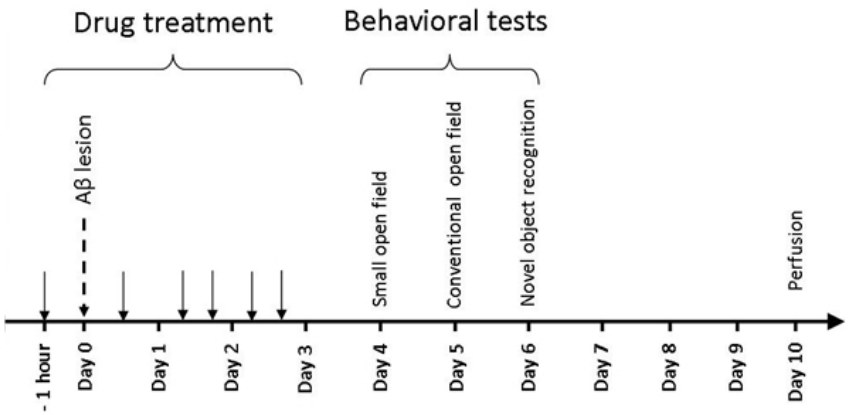

Figure 5.1. *Schematic outline of the experimental setup for the in vivo experiments. Rats received in total 6 intraperitoneal injections of the calpain inhibitor A-705253 or saline. The first injection was given 1 hour prior to unilateral Aβ lesions into the nucleus basalis magnocellularis, the other 5 injections followed within two consecutive days after the lesion. From the fourth day on (after the lesion) the rats were subjected to different behavioral tests. Ten days after the lesion the animals were transcardially perfused and the brains removed for immunohistochemical analysis.*

5.2.6 Novel Object Recognition

Testing the ability of rats to recognize a novel object in an otherwise familiar environment represents a sensitive and discriminating test to assess memory performance. Novel object recognition was measured in a conventional cylindrical open-field box of 80 cm diameter, in which the floor was divided into 20 sectors and surrounded by a 60 cm high reflective aluminum wall (Nyakas *et al.*, 2009).
During the first day the rats were allowed to habituate to the apparatus and their open-field behavior was recorded for 3 min (conventional open field). The test was carried out the day following to the 3 min open-field test and consisted of 2 sessions. During the 1st session the rats were allowed to explore in the open-field apparatus for 5 min while two identical objects (A + A) were placed into the arena. During the 2nd session, carried out 2.5 hrs after the 1st session, one of the two objects was replaced by a novel one (B + A). The ratio of visiting the novel versus known objects indicated the object recognition ability, i.e. attention behavior. The number and the period in sec spent with exploration of the objects during the 1st and 2nd sessions were recorded. The recognition ability of the novel object at the 2nd session was calculated in the following way: duration of exploration of the novel (B) object was divided by the duration of exploration of both novel (B) and familiar (A)

objects and expressed in percentage. The criterion to pass the test was that both objects had to be visited at least 5 times during the first session representing sufficient interest for object exploration. From the 57 animals only one failed to perform the test, thus statistical analysis was conducted on 56 animals.

5.2.7 Brain tissue processing

At postoperative day 10 the animals were transcardially perfused under deep pentobarbital anesthesia with a fixative solution of 4 % paraformaldehyde in 0.1 M phosphate buffer pH 7.4 after a short pre-rinse with heparinized saline. The brains were removed, post fixed for two days in the same fixative and stored in phosphate buffer at 0 °C, cryoprotected by 30 % sucrose for 4 days and sectioned on a cryostat microtome at a thickness of 20 μm. Vials processed for immunostaining contained every 10th section at the level of NBM, i.e. the serial sections positioned 200 μm apart. Free floating brain sections were processed for immunocytochemical staining.

5.2.8 ChAT staining of cholinergic fibers

Immunostaining procedure was applied to visualize choline acetyltransferase (ChAT) positive cholinergic neurons in NBM and their axon ramifications in the target brain areas selecting parietal neocortex. Goat anti ChAT primary antibody (AB144P, Lot: LV1359401; Chemicon International, Temecula, CA, USA) was used in dilution 1:500. Biotinylated rabbit anti goat antibody and Vectastain ACB kit was obtained from Vector Laboratories (CA, USA). The staining was completed with nickel-enhanced diaminobenzidine (DAB) reaction in the presence of H_2O_2.

5.2.9 Microglial activation

Mouse anti rat integrin αM [CD11b] monoclonal antibody (CLB1512, Lot: 0604026553; Chemicon International, Temecula, CA, USA) was used as first antibody in a dilution rate of 1:1500 to recognize activated microglia. The biotinylated second antibody and the ABC kit were obtained from Vector Labs (Ca, USA, see above). DAB reaction was enhanced by nickel ammonium sulfate.

5.2.10 Quantification of cortical cholinergic innervation

Reduction in cholinergic fiber density and cholinergic neurons

The quantification procedure for cholinergic fiber density in the parietal neocortex was established in our laboratory and described in detail in a series of previous publications (Horvath *et al.*, 2000; Harkany *et al.*, 2001; Dolga *et al.*, 2009). Briefly, parietal neocortex which is topographically the target of afferent cholinergic pathway from the NBM sites where the $A\beta_{42}$ oligomers were injected, was analyzed for ChAT positive fiber density with a Quantimet 600HR (Leica, Germany) image

analysis program. The exact measurement took place in the superficial sublayer of the layer V cortical area representing the densest zone of cortical cholinergic innervation. In addition we determined the number of ChAT positive neurons in the NBM. Three brain sections were analyzed for each experimental animal and the results were averaged. Percent surface area of positively stained fibers against zero background and number of positively stained neurons were computed in both sides of the brain section. The ChAT positive fiber density and NBM cell count ipsilaterally to the lesion was compared to the intact contralateral side and the percent decrement was calculated as an indicator of cholinergic degeneration.

5.2.11 Magnitude of microglial reaction

The magnitude of microglial activation was quantified with by image analysis measuring the extent of CD11b-immunoreactive microglia in a specific volume at the level of the lesion site (total infiltration volume). Therefore, a series of positively stained sections were used to reconstruct the extent of activation area. At each section the surface area of core structure around the injection channel and the size of infiltration area were measured with the Quantimet 600 system (Leica, Germany) by means of manual delineation of the affected area. Based on these measurements the total volume of the infiltrated brain area was computed: $(x_1+x_2+...x_n) \cdot 200$ µm; where 'x' was the cross-sectioned stained area in μm^2 and 200 µm was the distance between two consecutive sections. The volume of affected brain area was expressed in μm^3.

5.2.12 Primary cortical neuron culture

Primary cortical neurons were prepared from embryonic brains (E14) of C57BL/6J mice. The cortices were carefully dissected, meninges were removed and the neurons separated by trituration. Cells were plated on poly-*D*-lysine pre-coated plates at a density of 1.2 x 10^5 cells/well (96 well plates). Neurobasal medium supplemented with 2 % (v/v) B27-supplement, 0.5 mM glutamine, 1 % (v/v) penicillin/streptomycin was used as a culture medium. After 48 h neurons were treated with 10 µM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged with fresh medium and after 6 days of *in vitro* culture, the neurons were used for experiments.

5.2.13 Treatment of cells

The neuroprotective effect of A-705253 was assessed by incubating neurons (cultured in 96 well plates) for 24 h with 25 µM or 50 µM oligomeric Aβ in the presence or absence of different concentrations of A-705253. After the treatment the cell viability was determined by an MTT-assay. All treatments were performed in triplicates and the experiments were repeated at least two times.

5.2.14 Determination of cell viability by MTT-assay

Neuronal viability was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide] assay as described previously (Mosman *et al.*, 1983). 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2 h of incubation, cells were lysed in DMSO. The absorbance of each well was measured with an automated ELISA plate reader (Bio-Rad, Munich, Germany) at 595 nm with a reference filter at 630 nm.

5.2.15 Statistics

The Statistica 8 package was applied for one-way ANOVA evaluation of the results of independent groups which was followed by Dunnett *post-hoc* t-test to reveal differences between two selected groups. The level of $p<0.05$ was accepted as significant. Data are presented as means ± S.E.M.

5.3 RESULTS

5.3.1 A-705253 protects neurons against Aβ-induced toxicity in vitro.

To investigate the neuroprotective properties of A-705253 against Aβ-induced toxicity, we treated primary cortical neurons with different concentrations (0.04 µM – 5 µM) of the calpain inhibitor and challenged with 25 µM or 50 µM of oligomeric $A\beta_{42}$ for 24 hours. Our results show that the calpain inhibitor A-705253 in the present condition protects primary cortical neurons against oligomeric $A\beta_{42}$. Moreover, we found that concentrations in the range of 1 µM of A-705253 in the current experimental setup is most effective (Figure 5.2A).

Furthermore, we were interested if the temporal dynamics of the treatment is important for the neuroprotective effect. Therefore we applied 1 µM A-705253 2h and 1 h before, 1 h and 2 h after, or together with the $A\beta_{42}$ challenge. We found that pre-treatment, or simultaneous treatment of A-705253 with $A\beta_{42}$ were fully protective against Aβ induced toxicity (Figure 5.2B). Interestingly, when neurons were treated one hour after the insult, calpain inhibition still exerted a significant neuroprotective effect. However, application of the compound could not prevent neuronal damage when applied 2 h after the insult.

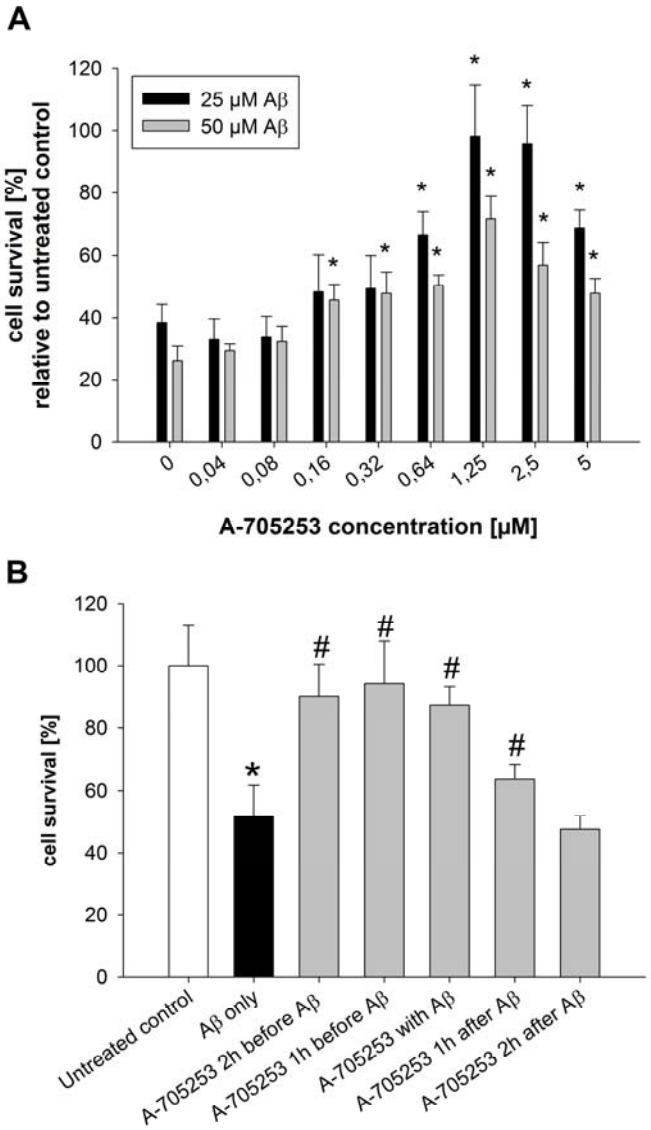

Figure 5.2. ***A)*** *Neuroprotection was determined by co-incubating increasing concentrations of calpain inhibitor A-705253 with 25 µM or 50 µM $A\beta_{42}$ for 24 h.* ***B)*** *The effect of the time point of treatment relative to the insult was assessed by incubating primary cortical neurons were with 1 µM A-705253 for 2h and 1h before, after or together with 20 µM oligomeric Aβ for 24 h. Cell viability determined by MTT-assay. Bars indicate the mean cell viability in % relative to untreated controls ± S.E.M. (*p < 0.05 vs untreated control and #p<0.05 vs Aβ only).*

5.3.2 Calpain inhibition prevents Aβ-induced degeneration of cholinergic fibers and neurons after lesion of the Nucleus Basalis Magnocellularis.

Recently, we demonstrated that the inhibition of calpain can protect cholinergic neurons against an NMDA-initiated excitotoxic insult (Nimmrich *et al.*, 2008). Although excitotoxicity is likely to contribute to the pathology of AD, this study did not reveal whether neuronal decline could also be prevented, if the insult was induced by Aβ.
To assess the sensitivity of cholinergic neurons to Aβ-induced toxicity we injected oligomeric $A\beta_{42}$ into the NBM and quantified the loss of cholinergic neurons and their cortical innervations that originate from the NBM (Figure 5.3) (Gaykema *et al.*, 1992). Cholinergic neurons and fibers were visualized with the cholinergic marker choline-acetyl transferase (ChAT, EC3.2.1.6) (Harkany *et al.*, 2000). The fiber density was measured in the superficial sublayer of the layer V as described earlier (Horvath *et al.*, 2000).

The cholinergic fiber density (Figure 5.3A and B) and the number of neurons (Figure 5.3C and D) ipsilaterally to the lesion was compared with the intact contralateral side respectively. The percentage of fiber and neuronal loss in the various experimental groups is summarized in Figure 5.4. Injection of oligomerized $A\beta_{42}$ into the NBM led to a loss of 40.6 ±2.3 % in cholinergic neurons, which is a 26.78 % higher depletion compared to sham injection (Figure 5.3C, D and 5.4A) and a concomitant decrease of 26.4±3.4 % in cholinergic fiber density (18.03 % compared to sham injected control) in the cortex (Figure 5.4B, C). Pre-treatment with 3 and 10 mg/kg of the calpain inhibitor A-705253 significantly prevented the Aβ-induced cell death and cholinergic fiber loss, respectively. The smallest dose of 1 mg/kg A-705253 was ineffective. The density of cholinergic neurons and fiber innervation was not affected in sham-treated animals, which received 10 mg/kg A-705253.

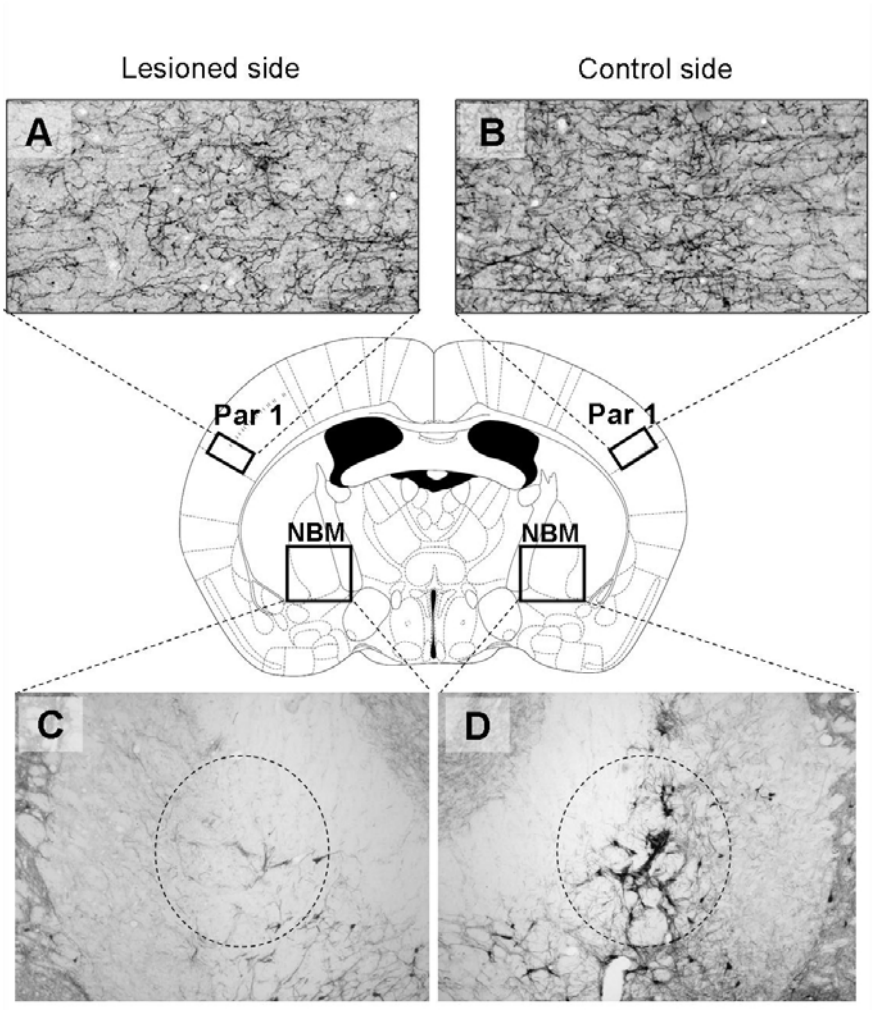

Figure 5.3*. Anatomical description of studying the cholinergic system after NBM lesion with oligomerized* $A\beta_{142}$ *. The upper panel shows ChAT positive fibers in the parietal neocortex of the lesioned rat.* ***A)*** $A\beta_{142}$ *lesioned side* ***B)*** *ipsilateral control side. The* $A\beta_{142}$ *injection site in the NBM (dashed circle) is depicted in figure* ***C)*** *the intact ipsilateral NBM (dashed circle) is shown in* ***D).***

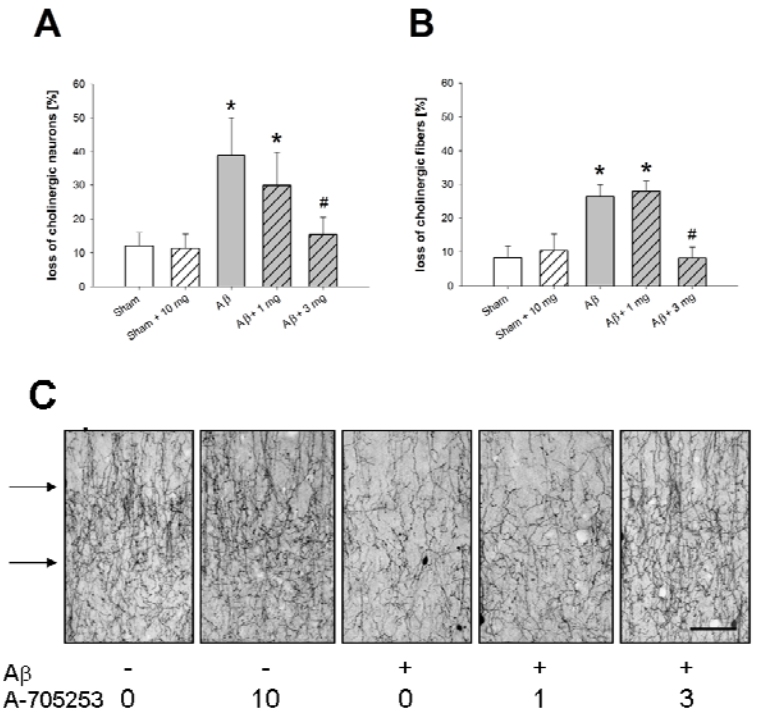

Figure 5.4*. Cholinergic neurons in the NBM and their innervation of the parietal neocortex was measured in 6 groups of animals: vehicle treated and sham-lesioned (Sham); sham-lesioned injected with 10mg/kg A-705253 (Sham + 10 mg); vehicle treated and $A\beta_{1-42}$-lesioned (Aβ); $A\beta_{1-42}$-lesioned and injected with A-705253 in doses of 1 and 3 mg/kg (labled Aβ + 1 or 3 respectively). **A)** $A\beta_{1-42}$ injection into the NBM resulted in decrease in cholinergic neurons. **B)** Decrease in cholinergic fiber density in the parietal neocortex after sham-injection and in the cholinergic NBM region. $A\beta_{1-42}$-injection (Aβ) increased the decline of cholinergic neurons and cortical innervation, which was not modulated by the smallest dose of 1 mg/kg, but greatly attenuated at 3 mg/kg. *p<0.01 vs. Sham; #p<0.01 vs. Ab. **C)** Representative photomicrographs of cholinergic innervation of the parietal neocortex. Quantitative measurement of fiber density was performed between the two arrows in the superficial part of layer V. The scale bar represents 100 μm.*

5.3.3 Reduced microglia activation in A-705253 treated rats.

In neurodegenerative diseases, microglial activation is an early sign that often precedes neuronal death and sustained local inflammatory responses. For that reason we determined the effect of calpain inhibition on microglia activation. We calculated the amount of CD11b immunoreactive microglia, as a measure of brain inflammation, in a specific volume at the level of the lesion site (infiltration volume) (Fig 5.5B). Injection of oligomerized $A\beta_{42}$ resulted in a strong activation of

microglia at the infusion site (Fig 5.5C), which was significantly reduced in animals treated with 3 mg/kg A-705253 as measured by CD11b expression (Fig 5.5A).

5.3.4 Calpain inhibition as well as NBM lesion does not affect explorative behavior in rats.

We further examined whether drug treatment or the Aβ-induced NBM lesion had an effect on the explorative behavior of the animals. Therefore, the rats were tested in a small open field and a conventional open field paradigm to asses explorative behavior. Neither the drug treated nor the Aβ-induced NBM lesioned animals showed any significant differences in behavior in the tested paradigms. Analysis of the different ambulatory behaviors in the open field tests, i.e. rearing walking, sniffing were not different from sham-treated rats. Exploration time, immobility as well as time spent grooming was also unaffected and reflects the behavior of healthy animals in such a paradigm (Figure 5.6 A-C).

5.3.5 A-705253 prevents Aβ-induced memory deficits in a novel object recognition paradigm.

Cholinergic neuronal projections from the NBM are well known to directly modulate neocortical attentional and memory functions. The novel object recognition task is a learning task which depends, in part, on proper neocortical information processing. In a previous study we could show that a lesion of the NBM in rats leads to attention and memory impairments in a novel object recognition paradigm (Nimmrich *et al.*, 2008). Therefore, we used this behavioral test to investigate whether the preservation of cortical cholinergic function by calpain inhibitor A-705253 can prevent the cognitive deficits induced by Aβ-mediated lesion of the NBM.

The novel object recognition test sensitively assesses attention, or discrimination ability between a novel object placed nearby to a familiar one in a habituated environment. The $A\beta_{42}$-lesioned group showed less attention towards the novel object as compared to the controls treated with vehicle. The amyloid-injected and drug treated groups performed all better than the lesioned control, and even the dose of 1 mg/kg A-705253 was effective in preventing $A\beta_{42}$-induced memory deficits (see Figure 5.6 D).

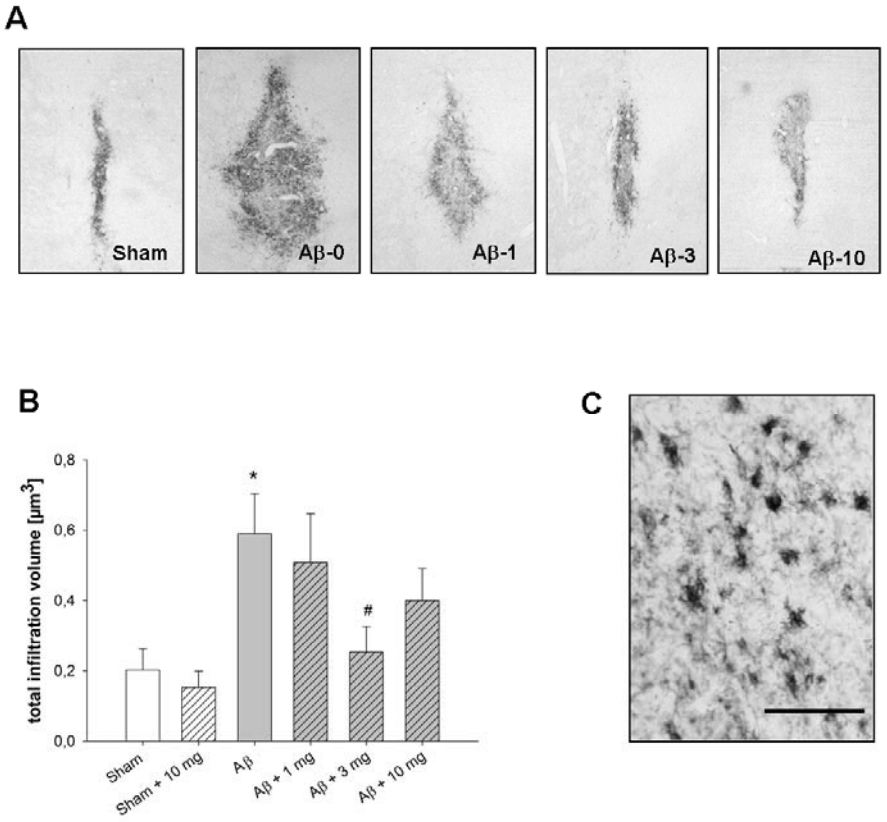

Figure 5.5. *Extent of microglia activation after $A\beta_{1-42}$ injection decreased under treatment with A-705253.* ***A)*** *Representative photomicrographs show the extent of microglia activation at the level of the brain injection site measured in 6 groups of animals: vehicle treated and sham-lesioned (Sham); sham-lesioned injected with 10mg/kg A-705253 (Sham + 10 mg); vehicle treated and $A\beta_{1-42}$-lesioned (Aβ); $A\beta_{1-42}$-lesioned and injected with A-705253 in three different doses of 1, 3 and 10 mg/kg (labled Aβ + 1, 3 or 10 respectively). The volume of total microglia infiltration volume around the injections (PBS or oligomers) is shown in* ***B)*** *(*p<0.05 vs. Sham and #p<0.05 vs. Aβ).* ***C)*** *High-magnification image shows robust microglia reaction around lesion site (scale bar 100 µm).*

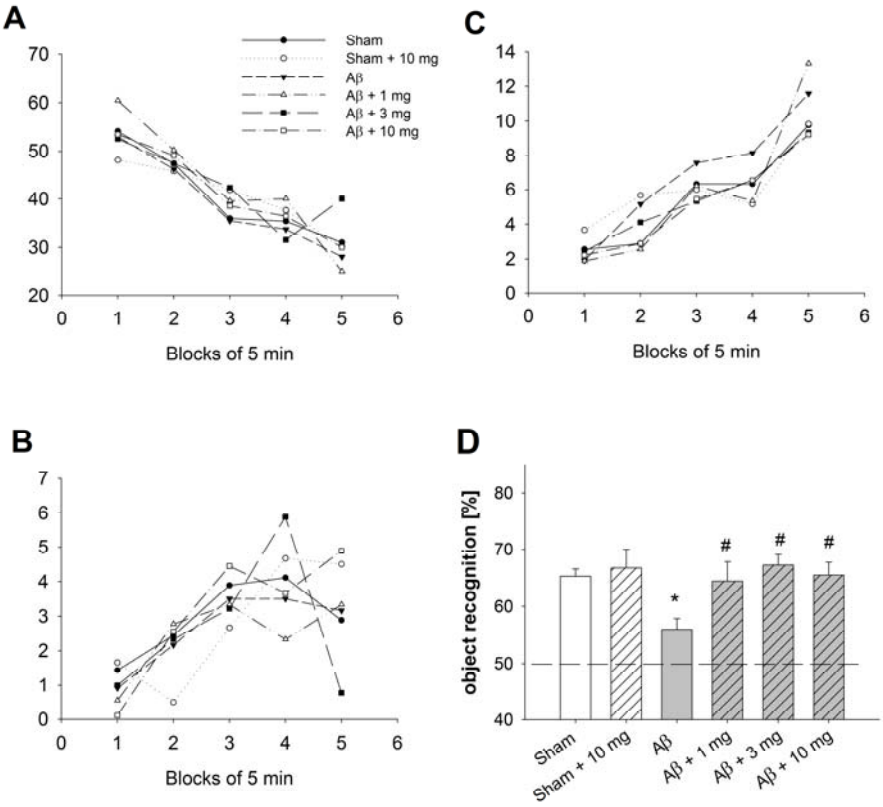

Figure 5.6*. Behavioral activity was compared in a small open field paradigm. Exploration is expressed as a combined score of rearing, walking and sniffing in* ***A),*** *grooming is depicted in* ***B)****, and immobility is shown in* ***C)****. Scores were collected by behavioral sampling every 10 s for 25 min. No statistical difference could be observed between the groups.*

Attention was estimated by measuring novel object recognition ability in a habituated open field. The results shown in ***D)****. Fifty percent performance means chance level since both the novel and familiar objects are explored by equal amount of time. Aβ_{1-42}-lesioned and vehicle-treated rats performed significantly worse than controls (Sham) (*$p<0.05$; Dunnett post hoc test). Treatment with different doses of A-705253 prevented the behavioral deficit caused by Aβ_{42}-injection (#$p<0.05$ vs. Aβ).*

5.4 DISCUSSION

Soluble, oligomeric forms of $A\beta_{42}$ are increasingly associated with the neuropathological mechanisms of AD. Whereas the toxicity of Aβ oligomers has been demonstrated in multiple studies, mainly *in vitro*, the mode of action of those peptide aggregates remains largely unknown. However, a greater understanding of the underlying process of amyloid toxicity is pressing in order to develop medication that specifically interferes with the Aβ-induced disease cascade. A number of authors reporting *in vitro* studies have suggested that the pathological effect of Aβ oligomers is mediated by the NMDA receptor (Shankar *et al.*, 2007; Kelly and Ferreira, 2006; DeFelice *et al.*, 2007), and that over activation of the NMDA receptor should be considered as a common principle for some major neurological diseases, including AD (Lipton and Rosenberg, 1994; Harkany *et al*, 2000; Mattson, 2004). Stimulation of the NMDA receptor leads to excessive entry of calcium into the cell, which activates proteases that are involved in cell death signaling. Calpain is a calcium-activated cysteine protease that has been implicated to contribute to NMDA-mediated excitotoxic cell death in various studies. For example, inhibition of calpain is neuroprotective after NMDA-exposure in hippocampal slice cultures (Caba *et al.*, 2002). *In vivo*, calpain inhibition reduces neurodegeneration in the rat retina after NMDA-injection (Chiu *et al*, 2005). Calpain has therefore been discussed as target for neurodegenerative disorders (Huang and Wang, 2001; Zatz and Starling, 2005; Saez *et al.*, 2006). Using the specific calpain inhibitor A-705253 we recently reported that inhibition of calpain prevents NMDA-induced lesioning of the NBM in rats, whereas physiological NMDA-cascades remained unaffected (Nimmrich *et al.*, 2008). However, although these studies suggested that calpain inhibition can prevent excitotoxic neurodegeneration in various *in vitro* and animal models, evidence that calpain inhibition could be protective against $A\beta_{42}$ oligomer-induced neuronal deterioration remained elusive.

The present study provides now evidence that calpain inhibition is neuroprotective against Aβ oligomer-induced cholinergic cell lesion. Injection of oligomeric Aβ to the nucleus basalis caused a strong deterioration of cortical cholinergic projections leading to deficits in learning behavior. Behavioral decline was prevented by application of the calpain inhibitor A-705253. The neuroprotective effect of calpain inhibition was also shown by morphological analysis: Cholinergic denervation in the parietal cortex and the extent of microglia activation around the injection in the NBM were greatly attenuated by A-705253. The present findings expand previous findings in animal models of excitotoxicity, and allow the conclusion that inhibition of calpain is effective *in vivo* against neurodegeneration triggered by oligomeric Aβ. Interestingly, our *in vitro* studies indicate that calpain inhibition is not only effective when the compound is applied prior to the insult. Efficacy of A-705253 against Aβ-induced cell damage was demonstrated – albeit less pronounced – up to 1 hour after administration of Aβ. This indicates that the Aβ-induced cell

death cascade – once initiated – may not instantly kill the neuron, but requires some time until degeneration is irreversible. If this holds true also for other acute forms of neurodegeneration, this could offer a promising avenue for the treatment of acute neurodegenerative disorders. It is likely that drugs targeting the NMDA receptor may only be effective up to the time of the insult. At least from studies on long-term potentiation (LTP), a neurophysiological paradigm considered to mimic neuronal memory processing, it is known that NMDA receptor-related cascades cannot be reversed by NMDAR blockers once the cascade is initiated. This could be the reason why patients suffering from acute neurodegenerative processes do not benefit from current pharmacological treatment: the drug is administered once the NMDAR-dependent cell death cascades have already started. Future studies need to reveal whether inhibition of calpain is also effective *in vivo* after NBM-lesioning, which is currently hampered by the fact that the time of Aβ diffusion to target cells cannot exactly be determined. The time point of treatment, however, may be less relevant for more chronic neurodegenerative diseases, like those accompanying amyloidoses. In any case, calpain inhibition might be superior to drugs targeting the NMDA receptor for another reason: calpain inhibition does not affect physiological processes like LTP (Nimmrich *et al.*, 2008). Thus, it is likely that calpain inhibition does not interfere with memory processes, but indeed contributes solely to the prevention of cognitive deficits. This is also indicated by the fact that the highest dose of A-705253 did not impair rat memory in several tasks (e.g. novel object recognition test) (Nimmrich *et al.*, 2008).

The compound used here was introduced by Lubish and co-workers (Lubish *et al.*, 2003) and does not affect caspases or the proteasome (Ki calpain=27 nM; Ki proteasome>10000 nM; Ki caspases>10000 nM). Therefore, it is likely that the protective effects against Aβ-oligomer toxicity are mediated by inhibition of calpain, and not by reducing proteasome or caspase activity. The proteasome complex (Rubinsztein, 2006; Pan *et al.*, 2008) as well as caspases (Cribbs *et al.*, 2004) potentially contribute to neurodegeneration, and a discrimination from these targets mandatory. It is likely that A-705253 has fully reached the target in our experiments as it rapidly penetrates tissues, affecting its cellular target within minutes after perfusion (Neuhof *et al.*, 2003; Neuhof *et al.*, 2004). It also effectively prevented Aβ-induced toxicity at doses previously shown to inhibit calpain (Nimmrich *et al.*, 2008). It should be noted that A-705253 also inhibits other molecules like cathepsin B to some extend. We therefore cannot fully exclude that at least some of the observed protective effects may be attributed to mechanisms other than calpain inhibition. In reverse, it is also possible that the reduction of efficacy at higher concentration is caused by inhibition of other proteins. Future synthesis of more selective compounds is highly desirable for dissecting the molecular components of neuroprotection.

The model presented here reflects some essential hallmarks of the AD pathology. The NBM is one of the early regions to be affected during AD and the overall

neuropathology in this model, including cholinergic denervation and microglial activation, features much of the pathology found in AD patients.

Beyond this, Aβ oligomers– rather than amyloid plaques or monomeric amyloid-β - are thought to underlie the neurotoxic initiator of the disease. They occur in patients prior to plaque formation and correlate much better with cognitive deficits than amyloid plaques. Aβ oligomers have been extracted from brains of AD patients and correlate well with the severity of the disease. Aβ oligomers are therefore – to the best of our knowledge – currently the most appropriate conformation of β-amyloid to induce pathology. The oligomer preparation used here has been introduced by Stine and colleagues (Stine *et al.*, 2003) and was shown to be toxic to neurons (Lambert *et al.*, 1998). They also impair LTP (Lambert *et al.*, 1998; Trommer *et al.*, 2004) and have been demonstrated to exhibit their pathogenic action via the NMDA receptor (Lacor *et al.*, 2007). The preparation is similar in their biophysical properties to brain derived Aβ oligomers, underlining their physiological relevance (Gong *et al.*, 2003). In spite of all similarities to AD it must be noted, however, that the experimental model presented here involves a rather rapid decline of neurons and thus differs from the slowly progressive neurodegeneration observed in AD. It would be challenging to examine whether calpain inhibition also prevents more chronic neurodegeneration processes, which is currently only feasible in transgenic mouse models. Most of these transgenic models, however, suffer from a lack of neurodegeneration and decline of cholinergic fibers. Hence, it may be an adequate approach to combine the effect of calpain inhibition on neurodegeneration in an acute *in vivo* model, and the prevention of other deficits – like synaptic decline – in chronic mouse models. The latter effects were explored by Battaglia and colleagues using another, less specific calpain inhibitor, E64 (Battaglia *et al.*, 2003), who showed that long-term (5 mo) intraperitoneal application of E64 in APP(K670N/ M671L)/PS1(M146L) mice prevented synaptic impairment as measured by LTP. Chronic calpain inhibition also attenuated the development of deficits in spatial behavior of these mice. Thus, it is likely that calpain inhibition serves to protect from acute and chronic neuronal decline, and from a variety of pathological features observed in AD.

Interestingly, protection from behavioral deficits in our study is more sensitive to calpain inhibition than the prevention of cholinergic denervation and microglia activation. A significant improvement in cognitive function measured by novel object recognition was already accomplished with 1 mg/Kg of A-705253 whereas the best protection from Aβ-induced cholinergic denervation was achieved with a dose of 3 mg/kg. This did not further improve with the highest dose of 10 mg/kg (data not shown). This suggests that the efficacy of calpain inhibitor A-705253 reaches a plateau at 3 mg/kg in our *in vivo* model.

In addition, the highest dose (10 mg/Kg) A-705253 did not significantly prevent microglia activation. This could indicate that neither cholinergic denervation nor

microglia activation do fully contribute to the behavioral outcome measured by the novel object recognition test. It is very likely that the microglia reaction might follow a different dose-response course, which might facilitate other molecular pathways, specific for microglia independent from functional changes behind the behavioural effects.
Whether the effect on behavior at 1 mg/kg may be due to an improvement of physiological function (and hence remain undetected by neuroanatomical analysis) should be assessed in further studies. Recent data showing the involvement of calpain in Aβ-mediated dysfunction of the synaptic vesicle machinery (Ferreira et al., 2005, Kelly and Ferreira, 2006) could support such view.
In summary the presented study add evidence that calpain inhibition significantly decreases acute Aβ oligomer-induced degeneration of NBM and its associated cholinergic fiber projections, and attenuates behavioral deficits associated with such lesions. Furthermore, *in vitro* data suggest that inhibition of calpain is still effective when performed shortly after the insult. Our findings indicate that inhibiting calpain could be a promising strategy for the therapeutic intervention of amyloid- β related neuropathology in AD.

CHAPTER 6

Inflammation and NF-κB in Alzheimer's disease and Diabetes

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ABSTRACT

Inflammatory processes are a hallmark of many chronic diseases including Alzheimer's disease and Diabetes mellitus. Fairly recent statistical evidence indicating that type two diabetes increases the risk of developing Alzheimer's disease has led to investigation of the potential common processes that could explain this relation. Here, we review the literature on how inflammation and the inducible nuclear factor NF- (kappa) B might be involved in both Diabetes mellitus and Alzheimer's disease, and whether these factors can link both diseases.

6.1 INTRODUCTION

Since the beginning of the 20th century, there has been a continuous knowledge-based improvement in healthcare in most industrialized societies. As a result, people in these affluent societies live longer, which has led to an increase in prevalence of age-related neurodegenerative diseases such as stroke and dementia. Diabetes mellitus (DM) has long been known as a risk factor for all vascular diseases, including vascular dementia. Indeed, the epidemiological relationship was recently explored in several large-scale studies. Furthermore, population-based cohort studies, such as the Rotterdam study have shown that patients with type 2 DM (T2DM) are approximately twice as likely to develop Alzheimer's disease (AD), independent of vascular factors (Leibson et al., 1997; Ott et al., 1999). The fact that neurodegenerative diseases are often associated with metabolic diseases such as DM suggests an underlying or aggravating metabolic basis. Here, we will provide a survey of the multiple levels of interactions between metabolic diseases and AD, and vice versa, and the particular role of inflammatory processes in these interactions. Although inflammation can be considered at least initially as a reaction of tissue against various stressors, chronic inflammation can be seen as part of the pathological process. A number of studies have shown both that inflammatory signaling in peripheral organs can influence central nervous system (CNS) functions, while reversely the CNS can have profound effects on peripheral functions. To partially illustrate these multiple mechanisms of interaction, we focus in this review on the transcription factor family NF-κB, which plays a major role in the initiation of the innate and adaptive immune response. This review concentrates on the role of inflammation and NF-κB in DM and AD.

6.1.1 Inflammation and Diabetes Mellitus

Involvement of inflammation in DM was first recognized more than a century ago, when Ebstein observed that the anti-inflammatory drug sodium salicylate dramatically reduced glucosuria in DM patients (Ebstein, 1876). Following the classification of type-1 (i.e., insulin-dependent) and type-2 (i.e., non-insulin-dependent) DM, other, more distinctive inflammatory processes were thought to play a role, especially in the aetiology of T2DM. T2DM, which is characterised by an insufficient release of insulin from pancreatic beta-cells to overcome insulin resistance in target tissues, is usually associated with increased levels of markers and mediators of inflammation and acute-phase reactants such as fibrinogen, C-reactive protein (CRP), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), sialic acid, and white cell count. An additional connection between inflammation, obesity, and T2DM in which obese patients exhibited an elevated level of cytokines that caused hepatic insulin resistance (Fig. 1) (Pradhan et al., 2001). In fact that T2DM is a comorbidity of increased visceral adiposity has stimulated interest in the role of adipose tissue as a mediator of inflammatory processes underlying the development of T2DM. Indeed, the proinflammatory cytokine tumor necrosis

factor- alpha (TNF-α), which is produced and secreted by adipose tissue, was found to induce insulin resistance through local and potentially systemic effects on metabolism (Feinstein et al., 1993; Hotamisligil et al., 1993). Subsequent to the idea that fat tissue was a site for the production of cytokines and other bioactive substances, the concept quickly developed beyond TNF-α to include leptin, IL-6, resistin, monocyte chemo attractant protein-1 (MCP-1), angiotensinogen, retinol-binding protein-4, serum amyloid A (SAA), and others (Fried et al., 1998; Steppan et al., 2001; Zhang et al., 1994). Furthermore, TNF-α, IL-6, MCP-1, visfatin, and PAI-1 are highly expressed in activated macrophages and/or other peripheral tissue, as well as in the brain. At this moment, it is not known if cytokines are produced by the adipocytes themselves or by monocytes which have invaded adipose tissue (Weisberg et al., 2003). This knowledge gap has engendered an intense debate.

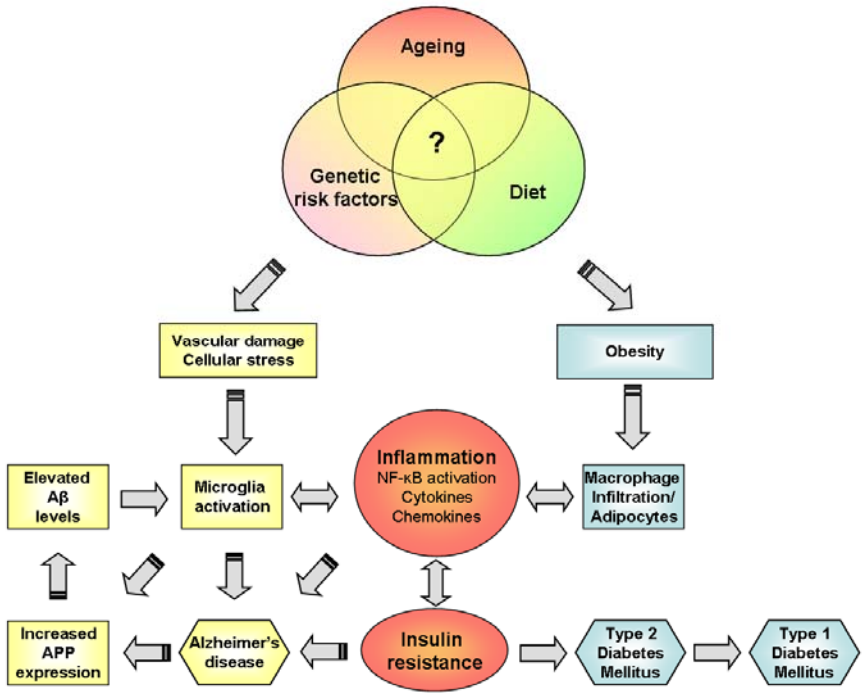

Figure 6.1. *Inflammation as a central event in the pathogenesis of AD and DM. Ageing, diet, genetic risk or other unknown factors can directly or indirectly lead to the pathological changes underlying both diseases. However, chronic inflammation may amplify the pathogenic processes through sustained NF-κB activation and elevated cytokine levels, which eventually lead to insulin resistance and DM, or result in increased Aβ production and microglia activation, which are symptomatic of AD.*

6.1.2 Inflammation in Alzheimer's Disease

The idea that neuroinflammatory factors play a role in the etiology of AD dates back to 1910, when Fischer reported that the extracellular deposition of a "foreign" substance in the cerebral cortex induced both a local inflammatory response and the formation of cerebral plaques (Fischer, 1910). Due to the inadequate detection methods at the time, it was not possible to identify the precise structure of this "foreign" substance or to establish the ultimate proof for the involvement of inflammatory molecules was. Today, however, it is well known that the major component of the "foreign" substances is the amyloid beta protein (Aβ). The Aβ peptide is the result of a proteolytic cleavage of the amyloid β precursor protein (AβPP). This precursor protein can be processed in two different ways: via amyloidogenic- or via non-amyloidogenic-processing. Non-amyloidogenic processing involves cleavage within the Aβ sequence by α-secretase, which prevents the formation of Aβ. On the other hand the amyloidogenic pathway is mediated by sequential cleavage of APP by β-secretase (BACE) and γ-secretase, which results in the generation of Aβ1-40 and Aβ1-42 species (Gandy, 2005; Mattson, 2004).
This Aβ peptide plays a central role in the neuroinflammation hypothesis of AD, which states that Aβ accumulation results in increased levels of inflammatory molecules (e.g. cytokines, chemokines, complement proteins) produced by chronically activated glia. This leads to neuronal damage, which in turn induces further glial activation, and results in a detrimental cycle of neuroinflammation and neurodegeneration (Griffin et al., 1998).

TNF-α is one of the most prominent pro-inflammatory cytokines significantly increased in AD and it plays a central role in initiating and regulating the cytokine cascade during inflammatory responses (Akiyama et al., 2000; Fillit et al., 1991). For example, TNF-α increases the expression of adhesion molecules on the vascular endothelium, which allows leukocytes and immune cells to infiltrate areas of tissue damage and infection (Perry et al., 2001). TNF-α exerts its biological functions via two distinct receptors: TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2). The 55 kDa TNF-R1 (p55/60) is a membrane-receptor and is expressed in most tissues where it can be stimulated by both the membrane-bound and the soluble form of TNF-α. The functions of TNF-R1 range from inducing apoptosis and differentiation to NF-κB-mediated cell survival (Hu, 2003). Similar to TNF-R1, also the 75 kDa TNF-R2 (p75/80) is a membrane-receptor, but because of its low affinity to soluble TNF-α it can be fully activated only by membrane-bound TNF-α. The functions of TNF-R2 are as complex as those of TNF-R1 and are still not revealed in all its detail. It is known that the action of the TNF receptors is strongly dependent on the cell type. For instance, TNF-R2 is able to amplify apoptotic signals from TNF-R1 in cancer cell lines (Wajant, 2003) but has also been reported to mediate neuroprotection, as shown in a model for glutamate-induced excitotoxicity (Marchetti et al., 2004). TNF-R2 exerts its protective properties when pre-stimulated with TNF-α, which suggests a neuroprotective role in the CNS (Dolga et al., 2008;

Marchetti et al., 2004). In AD patients, TNF-R1 levels are increased (Fillit et al., 1991), whereas TNF-R2 (Taoufik et al., 2007) levels are decreased.
Recently, it was demonstrated that overexpression of TNF-R1 promotes Aβ-induced neuronal death in an APP overexpressing mouse model for AD (Li et al., 2004). In contrast, such mice lacking TNF-R1 have a decreased amyloid plaque burden, lower expression of β-secretase (BACE), and improved learning abilities compared to controls (He et al., 2007). Interestingly, the stimulation of both TNF receptors can lead to the activation of NF-κB, which has binding sites in the promoter regions of both the APP and the BACE gene (Grilli et al., 1996). Mutations in the NF-κB promotor region of BACE lead to a significant decrease in promotor activity of TNF-α activated glia cells or Aβ exposed neurons, which indicates an activating role of NF-κB in BACE expression (Bourne et al., 2007). In this way, NF-κB activation can lead to increased APP expression and enhanced amyloidogenic APP processing. Elevated APP and BACE expression will ultimately lead to increased Aβ production, which can in turn activate glia cells and enhance neuroinflammatory processes.

6.1.3 Inflammation and the NF-κB Family

Inflammation is defined as the local response to tissue injury (Cone, 2001), and NF-κB is considered as a primary regulator of inflammatory processes. The NF-κB family of transcription factors is an evolutionarily conserved signalling system that plays an important role in many biological processes in addition to inflammation. NF-κB was first described in B cells, but it was later shown to exist in an inactive cytosolic form in all cell types, including the nervous system (O'Neill and Kaltschmidt, 1997). NF-κB also plays a central role in the initiation and amplification of inflammation by responding to proinflammatory stimuli such as TNF-α or interleukin-1 (IL-1) (Hayden et al., 2006; Karin and Greten, 2005; Tak and Firestein, 2001). As illustrated in Figure 6.1, aberrant regulation of NF-κB leads to the development of many pathological states especially those involving acute inflammation such as AD and DM (Kaltschmidt et al., 1997; Katarina et al., 2007).
However, NF-κB is not the only transcription factor activated under inflammatory conditions. Activation of other transcription factors such as PPARγ and STAT-1 have also been implicated in AD (Sastre et al., 2006) (Cho et al., 2007).

6.2 Involvement of NF-κB in Diabetes Mellitus and Alzheimer's Disease

6.2.1 The NF-κB family

Different members of the NF-κB family have been identified in mammalian cells: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). NF-κB1 and NF-κB2 are synthesized as large precursors, p105 and p100, which are post-translationally processed to the DNA-binding subunits p50 and p52, respectively. All members of the NF-κB family have an N-terminal 300 amino acid Rel homology domain that allows DNA binding, dimerization, and nuclear localization in common (Baeuerle and Henkel, 1994). NF-κB proteins are present in unstimulated cells such as homo- or heterodimers bound to the inhibitor of the kAPP a B (IκB) family of proteins IκBα, IκBβ, IκBγ, IκBδ, IκBNS, Bcl-3, and the p100 and p105 precursor proteins. Association with IκB prevents the nuclear translocation of the NF-κB:IκB complex.

Currently, two NF-κB activation pathways have been identified (Fig. 2). Classical/canonical NF-κB activity is stimulated by proinflammatory cytokines, such as TNF-α and IL-1, as well as by pathogen-associated molecular patterns (PAMPs). The binding of these ligands to their respective receptors, i.e. the TNF-R, Toll-like receptor (TLR) and the interleukin-1 (IL-1) receptor (IL-1R) superfamilies, causes activation of various cellular signaling pathways such as the protein kinase C (PKC), mitogen-activated protein (MAP) kinase kinase kinase-1 (MEKK1), and also the PKB/Akt signaling pathway, all of which have the potential to phosphorylate the IκB kinase (IKK) complex. IKK consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ). In the canonical pathway, the activated IKK complex predominantly acts through IKKβ in an IKKγ-dependent manner to catalyze the phosphorylation of IκBs. Upon phosphorylation, IκB releases NF-κB, allowing it to translocate to the nucleus and to initiate gene transcription (Figure 6.2a). The most commonly released NF-κB dimer in this pathway is the p50–RelA dimer (Ghosh and Karin, 2002).

The alternative/non-canonical activation pathway is activated by certain members of the TNF cytokine family but not by TNF-α itself and is strictly dependent on IKKα (Senftleben et al., 2001). The target for IKKα homodimers in this pathway is NF-κB2/p100 (Figure 6.2b).

Recent results strongly suggest that the canonical and non-canonical pathways to NF-κB activation have distinct regulatory functions. Whereas the classical pathway was found to be mostly involved in innate immunity and maintaining survival of immune cells, the alternative pathway is an important player in adaptive immunity and the development and organization of secondary lymphoid organs and B-cell maturation (Bonizzi and Karin, 2004).

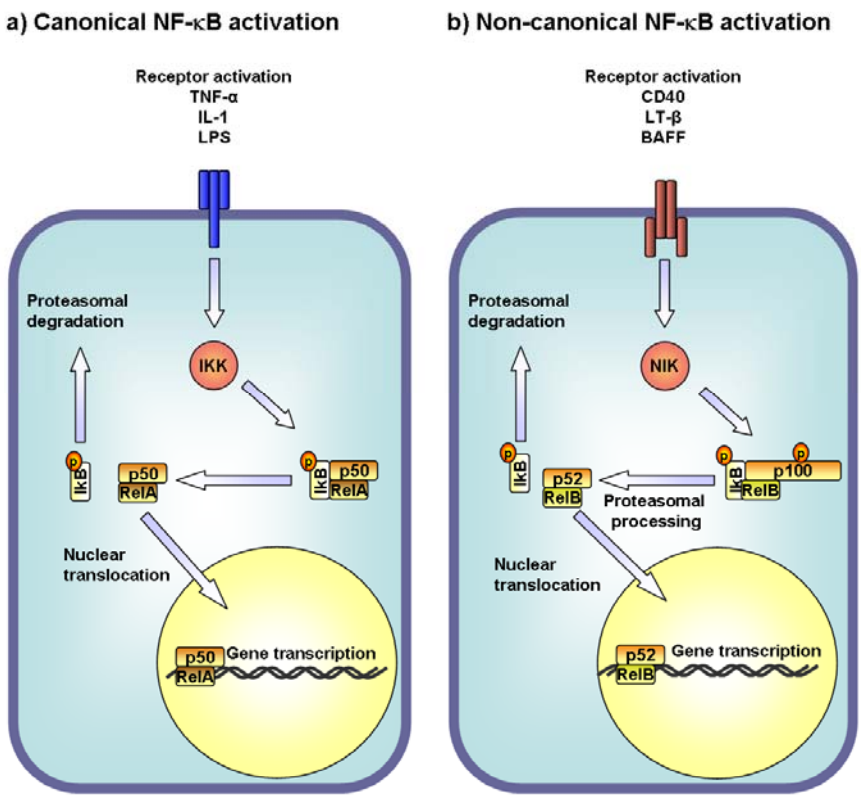

Figure 6.2. *The activation of NF-κB transcription factors occurs through two main pathways: a) the canonical and b) the non-canonical NF-κB activation pathway. During canonical signalling upstream mediators activate the IκB kinase (IKK) complex to phosphorylate inhibitory IκB proteins, leading to their ubiquitination and degradation. NF-κB dimers are then free to bind to DNA and to activate gene transcription. Alternative, non-canonical NF-κB signalling is mediated by NF-κB Inducing Kinase (NIK), which induces the partial proteolytical degradation of p100 to p52. RelB, together with p52, translocates to the nucleus and transactivates their target genes.*

NF-κB regulates several promoters containing variations in a highly divergent consensus DNA-binding sequence. Variations in the DNA-binding site APP ear to confer regulatory specificity for NF-κB family members by two general mechanisms. The sequence of the site can determine which coactivators form productive interactions with the bound NF-κB dimer (Leung et al., 2004). This mode of specificity occurs independently of any inherent difference in DNA binding by distinct dimers. A second mechanism conferring specificity of transcriptional regulation involves differential affinity of NF-κB dimer combinations for different DNA-binding sequences sites. NF-κB signalling can be switched off through multiple mechanisms, including the new synthesis of IκBα protein.

The activation and nuclear translocation of classical NF-κB dimers (mostly p50–RelA) is associated with increased transcription of genes encoding chemokines, cytokines, adhesion molecules [intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial–leukocyte adhesion molecule 1 (ELAM)], all of which are factors that produce secondary inflammatory mediators (Ghosh et al., 1998). NF-κB regulates proliferation and apoptosis by controlling the expression of the cellular inhibitors of apoptosis (cIAP1, cIAP2, and XIAP) (Deveraux and Reed, 1999); (Wang et al., 1998), the TNF receptor associated factors (TRAF1 and TRAF2) (Wang et al., 1998), the bcl-2 homologue A1/Bfl-1, and IEX-IL (Wu et al., 1998). Interestingly, NF-κB activation can thus lead to the expression of the same cytokines that can also regulate its activity, such as IL-1β and TNF-α. This results in a positive auto-regulatory loop contributing to the amplification of the inflammatory response and the persistence of chronic inflammation at local sites.

6.2.2 NF-κB and Diabetes

After Ebstein's discovery (Ebstein, 1876) and somewhat later investigation by Williamson (Williamson, 1901), the role of salicylates in the treatment DM were long forgotten, in part because the fact that the well-established effects of low doses of salicylates to block cyclo-oxygenase enzyme activation of prostaglandin synthesis (Simmons et al., 2004), were far below the concentration of salicylates necessary to induce effects on glucose homeostasis (Mitchell et al., 1997). High doses of salicylates, however, had been found to inhibit NF-κB (Mitchell et al., 1997), and its upstream component, IκB kinase-β (IKK-β). Cai and colleagues (Cai et al., 2005) were able to induce a T2DM phenotype in mice by selectively expressing IKKβ in hepatocytes, characterized by hyperglycemia and insulin resistance. A definitive role for the IKKβ/NF-κB in glucose homeostasis was substantiated when targeted deletion of IKK-β in obese and diabetic mice completely reversed insulin resistance in these animals (Hundal et al., 2002; Shoelson et al., 2003; Yuan et al., 2001).

Evidence in support of a critical role of NF-κB in the pathogenesis in early stages of DM can be described as threefold (regarded as activators, effectors, and as modulators of the NF-κB activity).

First, in DM, the increase in NF-κB activators promotes vascular complications. Thus far, at least four molecular mechanisms have been implicated in glucose-mediated vascular diseases. These mechanisms include: glucose-mediated activation of protein kinase C (PKC) isoforms; increased formation of glucose-derived advanced glycation endproducts (AGEs); the aldose-reductase (AR) pathway; and increased formation of reactive oxygen species (ROS). Furthermore, all of these pathways have been demonstrated to increase NF-κB activity. Hyperglycemia leads to the activation of different isoforms of PKC in accordance with tissue types, thus determining the specific kind of DM-induced organ damage (Ibrahim et al., 2008).

Activation of the AR pathway is necessary for high-glucose-induced TNF-α synthesis and release. In DM, high-glucose mediated TNF-α release has been shown to cause cardiovascular complications (Ramana et al., 2007). Although targeting PKC isoforms and AR products has proven to be a difficult and challenging task, targeting glucose-derived AGE- receptor for advanced glycation endproducts (RAGE) products became more feasible with the availability of RAGE knockout mice and a competitive decoy for AGEs, soluble RAGE (sRAGE). Diabetic RAGE overexpressing transgenic mice showed exacerbated neuropathy, while inhibition of AGE formation prevented these vascular cell derangements (Bierhaus et al., 2004; Toth et al., 2008). On the the other hand, it was found that RAGE knockout mice were resistant to DM-induced neuropathy. In human DM patients, NF-κB activation is a time consuming process and is associated with increased transcription of the p65 subunit of NF-κB (Bierhaus et al., 2001). These findings were paralleled by *in vitro* studies, in which it was shown that RAGE-expressing cells induced sustained translocation of p50/p65 subunits of NF-κB from the cytoplasm into the nucleus. RAGE ligands induce NF-κB activation by initial degradation of IκB proteins, followed by new synthesis of the p65 subunit of NF-κB, although IκBα and IκBβ are also generated. These findings indicate an important function of *de novo* synthesized p65 subunit in prevailing the NF-κB auto-inhibitory effects, thus inducing a sustained NF-κB activation in hyperglycemic conditions (Bierhaus et al., 2001). Several reports have shown that IKKβ, which is responsible for the activation of NF-κB, was also increased in insulin-resistant cells (Yuan et al., 2001) (Shoelson et al., 2003).
The importance of IKKβ in insulin resistance was provided by data accumulated from IKKβ (+/-) heterozygous mice. Whereas IKKβ (-/-) homozygous mice died in embryonic stages due to massive apoptosis in the liver, IKKβ (+/-) heterozygous mice were reported to exhibit a stronger insulin sensitivity compared with their littermates (Yuan et al., 2001).

Second, inhibition of NF-κB effectors, such as iNOS or ICAM proteins mediates beneficial effects reducing DM-induced degeneration of retinal capillaries (Chu and Ali, 2008). In retinas of mice exhibiting streptozotocin-induced DM, the concentrations of NO, of iNOS, the nitration of proteins and leukostasis were significantly increased when compared with non-diabetic mice (Zheng et al., 2007). Interestingly, in diabetic iNOS-/- mice not all of the above-mentioned abnormalities were detected. In addition, retinas from stretozotocin-induced DM mice were significantly thinner than from non-diabetic control mice, whereas no retinal defects were observed in diabetic iNOS -/- mice. Administration of the iNOS specific inhibitor, N(G)-nitro-L-arginine methyl ester, reduced DM-mediated leukostatis within retinal vessels and blood-retinal barrier permeability (Leal et al., 2007). These studies showed that iNOS enzymes play a crucial role in the pathogenesis of vascular lesions detected in early stages of diabetic retinopathy (Leal et al., 2007; Zheng et al., 2007).

Third, compounds known to inhibit the NF-κB pathway also inhibit the development of the DM retinopathy or neuropathy. In addition to the augmented oxidative stress-mediated cellular damage, type 2 DM patients usually present defects of cellular antioxidant defense mechanisms, such as the glutathione redox system, vitamin C-vitamin E cycle, and the α-lipoic acid (LA)/dihydrolipoic acid (DHLA) redox pair (Evans et al., 2002). Antioxidants inhibit the activation of NF-κB and the development of inflammatory responses in several tissues, including retinas of diabetic animals (Kowluru et al., 2003). Consistent with these findings, treatment with antioxidants significantly suppressed the NF-κB activity and reduced plasma markers for lipid oxidation (Hofmann et al., 1998). LA treatment of diabetic neuropathy restored the LA pool, increased the insulin sensitivity (Jacob et al., 1999), raised intracellular glutathione levels (Han et al., 1997), prevented glycation of serum albumin (Biewenga et al., 1997) and reduced the oxidative stress-mediated NF- κB activation (Hofmann et al., 1998).

6.2.3 NF-κB in Alzheimer's disease

NF-κB activation as a central event of inflammation is a common feature of many neurodegenerative diseases such as Huntington, Parkinson, stroke, and particularly of AD. In the brain of AD patients, activated NF-κB was found predominantly in neurons and glial cells in Aβ plaque surrounding areas (Boissiere et al., 1997; Kaltschmidt et al., 1997; Lukiw and Bazan, 1998; Terai et al., 1996). The reactive astrocytes in close proximity to the Aβ plaques produce inflammatory cytokines, including IL-1b and TNF-α, and iNOS, which generates free radicals such as NO, that can be neurotoxic (Akama et al., 1998; Lee and Brosnan, 1996). Several studies have shown that Aβ and/or a secreted form of APP induce an up-regulation of NF-κB activity. Some non-steroidal anti-inflammatory drugs (NSAIDs) have a direct effect on NF-κB activity, which eventually results in decreased APP processing. Flurbiprofen and indomethacin, which target NF-κB, have been shown to effectively reduce the amyloid load *in vitro* and also in APP transgenic mice (Eriksen et al., 2003; Sung et al., 2004). The activation of NF-κB leads to the expression of a large variety of pro-inflammatory molecules such as cytokines and chemokines, which could be in partly responsible for the neurotoxicity seen in AD. However, there are also reports that certain cytokines, e.g. TNF-α, may trigger NF-κB activation, which seems to be neuroprotective against Aβ toxicity in cultured neurons (Barger et al., 1995; Barger and Mattson, 1996).
Both pathological hallmarks of AD (Aβ and hyperphosphorylated tau) are capable to inducing NF-κB activation via various mechanisms. One common mechanism is the activation of the AGE/RAGE signalling pathway. Aβ and tau can undergo a non-enzymatic glycation and form AGEs. These AGEs, which bind to so-called RAGEs and can trigger NF-κB dependent gene transcription. Furthermore, glycation of Aβ enhances its aggregation in vitro (Vitek et al., 1994), and glycated tau, in addition to hyperphosphorylation, APP ears to enhance the formation of paired helical filaments (PHFs) (Ledesma et al., 1994; Yan et al., 1994). In addition,

AGEs have been reported to generate reactive oxygen intermediates, leading to the activation of cytokines including IL-1β and TNF-α, which in turn induce the translocation of NF-κB to the nucleus (Yan et al., 1995).
The recently discovered prolyl isomerase protein Pin1, which accelerates the *trans* to *cis* or *cis* to *trans* isomerization of target proteins, is APP arently a 'key player' in the pathogenesis of AD (Lu et al., 1999; Pastorino et al., 2006). Pin1 has been shown to bind to both APP and phosphorylated tau, where it stabilizes the non-pathogenic conformations of both proteins. In AD, Pin1 levels are compromized, leading to increased Aβ formation and reduced tau de-phosphorylation. In addition, Pin1 might have a role in neuronal apoptosis of AD affected cells as well, either indirectly, via depletion in the levels or aberrant function of Pin1 (resulting from oxidation, phosphorylation or mutations) or directly, via association with specific signaling proteins such as NF-κB.
During TNF-α treatment, Pin1 binds to the phosphorylated p65/Rel subunit on Thr 254 (Ryo et al., 2003), enhancing NF-κB DNA binding and transactivation activity. Pin1 binding to p65/Rel inhibits p65 binding to IκBα, which prevents p65/Rel nuclear export and its subsequent ubiquitin mediated degradation. This leads to enhanced nuclear accumulation, protein stability, and transcriptional activity of NF-κB towards its target genes, which in turn promotes neuronal survival (Ryo et al., 2003).
Furthermore, a single mutation in p65/Rel abolishes Pin1 binding and destabilizes the protein in HeLa and 293 cells. The source of destabilization has been found to be enhanced ubiquitin mediated proteasomal degradation (Ryo et al., 2003). Ryo and colleagues (Ryo et al., 2003) suggest that Pin1 stabilizes p65 and prevents its proteasomal degradation, probably by isomerizing the Thr254-Pro motif towards a more stable conformation, possibly indicating that in neurons in which NF-κB is induced, Pin1 might mediates neuronal survival by stabilizing NF-κB, resulting in the transcription of its pro-survival target genes. In addition, Pin1 has also been shown to bind to these anti-apoptotic NF-κB target genes, possibly stimulating survival downstream of NF-κB signaling as well (Lu et al., 2002). Thus, depletion of Pin1 could accelerate neuronal cell death in AD patients. Since stabilization of the p65/Rel subunit of NF-κB by Pin1 no longer takes place, it will ultimately lead to a down regulation of anti-apoptotic genes and, ultimately, to cellular death.

6.3 Mechanisms relating Type-2 Diabetes Mellitus and Alzheimer's Disease

Since the Rotterdam study and other studies linked DM with an increased risk to develop AD, researchers worldwide have searched for the causal mechanisms underlying this relationship. Becaus the NF-κB pathway plays a major role in the aetiology in both diseases, it seems reasonable to assume that mechanisms causing disturbances in this pathway are at the core of the relationship (Figure 6.3). A number of issues relevant to this possibility need to be addressed.

First, in addition, to their role in inflammation and immune responses, neurons and their neighbouring glial cells also employ the NF-κB pathway for distinctive functions ranging from the development to the coordination of cellular responses to injury of the nervous system, and to brain-specific processes such as the synaptic signaling that underlies learning and memory. Interestingly, electrical activity within neurons and synaptic transmission between neurons are potent stimuli for NF-κB activation, and such neuronal activity may account for the relatively high constitutive activity of NF-κB in brain tissue compared with other tissues (O'Neill and Kaltschmidt, 1997). As in other organs, NF-κB influences the expression of a complex array of genes in the nervous system, and, in general, these genes serve important functions in cellular responses to injury and in neuronal plasticity. In the nervous system, the cell type and the duration of NF-κB activation APP ears to be a determining factor for the output of NF-κB in neurodegenerative diseases such as AD. NF-κB activation in neurons has been shown to promote the neurons' survival and plasticity. On the other hand NF-κB activation in glial cells may play a major role in inflammatory processes that can damage and kill neurons. Indeed, in some cases, the NF-κB and the NF-κB responsive genes may serve dual functions. In neuronal cells sustained activation of NF-κB has been shown to induce neuroprotection via PI3-kinase-PKB/Akt pathway activation (Marchetti et al., 2004). Furthermore, NF-κB cellular survival functions underlie cytokine-induced neuroprotective mechanisms, including transforming growth factor-beta1 (TGF-β1) and TNF-α. Although transient activation of NF-κB in activated glial cells is beneficial for defence processes, either chronic activation or overactivation may exacerbate neuronal diseases as seen in AD (Guzman and Blazquez, 2001; Kreutzberg, 1996).

The second issue relates to the use of substrates for metabolic purposes. Although the brain is preferentially utilizes glucose as the main substrate, ketone bodies may replace glucose as the major energy source. The liver is the main source for elevated ketones in the circulation (e.g. in starvation or hypoglycemia), but glial cells are also suitable to engage in ketone body production via stimulation of AMP-activated protein kinase (AMPK), a highly conserved stress-activated kinase (Blazquez et al., 1999; Guzman and Blazquez, 2001). Sustained glucoprivation and hypoxia, however, may cause neurons to release glutamate, which in turn leads to chronic activation of the NF-κB pathway in glia cells and their pro-inflammatory actions.
The third issue relates to the fact that glial cells require insulin for glucose uptake (i.e, which is comparable to peripheral tissue), whereas neuronal glucose uptake is independent of insulin (Wei and Yeh, 1991). Thus, in the case of insulin resistance, which is underlies T2DM, this would result in a limitation of glucose uptake in glial cells but not in neurons. In fact, in the case of T2DM, neurons often evidence elevated levels of glucose, which may cause direct deleterious effects via the RAGE pathway (Ramasamy et al., 2005). A reduction in glucose availability at the level of glial cells might be expected to cause augmented ketone body production and the chronic activation of the NF-κB pathway with the resulting

inflammatory assaults on neuronal tissue, a scenario is somewhat comparable to the recent discussion by Erol (Erol, 2008). Here, we suggest a more central role for the NF-κB pathway than has been put forward previously. Whether this scenario can explain the causality, however, is still under investigation. Moroz and colleagues recently found that mice chronically subjected to a high-fat diet displayed all the characteristics of peripheral and central insulin resistance, but signs of AD were only marginally observed (Moroz et al., 2008). Future experiments must address species differences (e.g., the variation of cholesterol metabolism in mice and in humans (MacLean et al., 2003)), timing and lifespan or ageing (Selman et al., 2008), and environmental challenges (e.g., psychological stress), of all which could act as additional factors interacting in the causal link between T2DM and AD. One particularly interesting permissive mechanism linking insulin and Aβ, which plays a central role in development of both diseases, is the insulin-degrading enzyme (IDE). In addition to defining a key role for IDE in both Aβ and insulin metabolism *in vivo*, selective deletion of the IDE gene recapitulates some of the hallmark phenotypic characteristics of AD and T2DM, namely chronic elevation of cerebral Aβ, as in AD, and hyperinsulinemia and glucose intolerance, as in T2DM (Farris et al., 2003). The state of chronic inflammation has great influence on the metabolism of Aβ and insulin. For example, in AD chronic inflammation and augmented NF-κB, activation leads eventually to elevated Aβ levels, activation of glia cells, and increased cytokine release. Furthermore, pro–inflammatory cytokines can directly reduce the expression of IDE (Yamamoto et al., 2008), which would compromise the clearance of Aβ and promote its aggregation. In T2DM, elevated levels of pro inflammatory cytokines such as TNF-α are known to induce insulin resistance in peripheral tissues by reducing insulin receptor substrate (IRS-1) phosphorylation (Peraldi and Spiegelman, 1997) (Figure 6.3).

Perez and colleagues (Perez et al., 2000) reported that the IDE activity may be reduced in AD patients compared to controls. IDE levels are reduced in diabetic APP transgenic mice that have increased cerebral Aβ levels compared to non diabetic APP transgenic controls (Ho et al., 2004). Clinical studies show that IDE levels are decreased in the hippocampi of AD patients who have the Apolipoprotein ε4 (Cook et al., 2003). In thist context, it is interesting to note that hippocampal volume is reduced in AD patients several years before any symptoms can be detected (Fox et al., 1996).

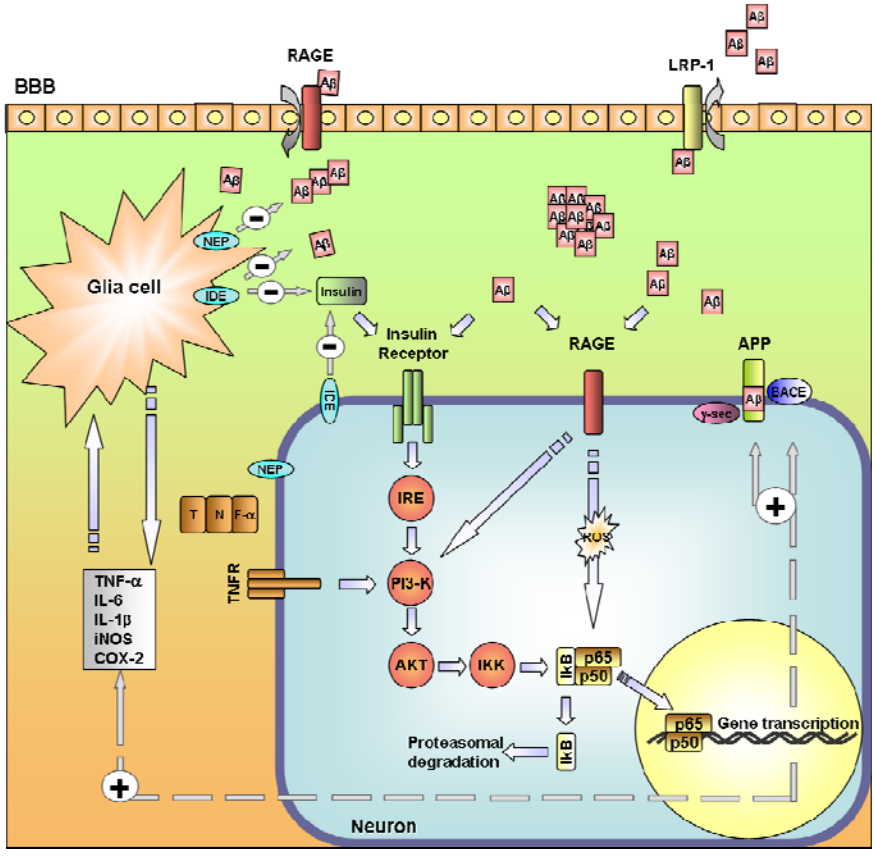

Figure 6.3. *Schematic representation of molecular pathways linking the pathogenesis of T2DM and AD. Conditions such as oxidative stress, pro-inflammatory cytokines, activated RAGE, and insulin receptor stimulation can directly or indirectly, lead to sustained activation of NF-κB (p50/p65). Upon phosphorylation IκB becomes degraded and NF-κB can translocate into the nucleus to initiate NF-κB dependent gene transcription (e.g. TNF-α, IL-6, IL-1β, iNOS, COX-2, BACE, APP). Aβ monomers and oligomers activate RAGE, whereas insulin and the Aβ protein compete for binding to the insulin receptor. Pro–inflammatory cytokines can directly reduce the expression of IDE, which would compromise the clearance of Aβ and insulin in the extracellular domain. Aβ, amyloid beta peptide; APP , Amyloid beta precursor protein; AKT, protein kinase B/AKT; BACE, beta-secretase; γsec, gamma-secretase; IDE insulin degrading enzyme; IKK, inhibitor of κB kinase; IκB, inhibitor of κB; IRE, insulin response element; LRP-1, Low density lipoprotein-related protein 1; NEP, neprilysine; PI3-K, phosphoinositide-3 kinase; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; TNF-α, tumor necrosis factor –alpha; TNF-R, TNF receptor; Dashed arrow: indirect effect; Full arrow: direct effect. +, positive stimulation; -, inhibitory effect.*

A recent paper showed that impaired insulin signaling can also lead to tau hyperphosphorylation in an animal model for DM (Planel et al., 2007). Insulin and insulin-like growth factor-1 stimulation reduces tau phosphorylation and promotes tau binding to microtubules. These effects of insulin and insulin-like growth factor-1 are mediated through inhibition of glycogen-synthase kinase-3 via the phosphatidylinositol 3-kinase/protein kinase B signaling pathway (Schubert et al., 2004). It is known that insulin and the Aβ protein compete for binding to the insulin receptor (Xie et al., 2002) Aβ, co-immunoprecipitates with the insulin receptor, and interferes with the autophosphorylation of the insulin receptor induced by insulin that affects downstream targets such as Erk/MAPK, CaMKII and PKB/Akt (Townsend et al., 2007; Zhao et al., 2008). Insulin resistance or hypoinsulinaemia cause elevated glucose levels which in turn leads to increases in AGEs. As the RAGE activates NF-κB, it is conceivable that such an inflammatory precondition might increase the risk for the onset of AD.
All of these mechanisms reflect the variety of links between T2DM and AD and how this linkage might lead to pre-conditions favouring the development of AD. However, it is also conceivable that central signal mechanisms preceding the phase of clinical symptoms of AD might also aggravate or favour T2DM .

6.4 Conclusions and future perspectives

It is clear from the reviewed literature that inflammatory processes are playing essential roles in the etiology of T2DM and AD. Aberrant regulation of the inflammatory pathway involving NF-κB, which includes TNF-α dependent (canonical) and TNF-α independent (non-canonical) mechanisms, directly underlies insulin resistance in peripheral tissue as well as in astrocytes in the brain. Since insulin resistance in the periphery can lead to glucose intolerance, neuronal inflammatory processes triggering (or triggered by) the NF-κB pathway may be propagated even further via stimulation of the AGE/RAGE signaling pathway. Therefore, it might be expected that alleviating symptoms of T2DM may be an effective way to treat AD. Indeed, recent clinical trials with the insulin sensitizer rosiglitazone -i.e., an agonist for the peroxisome proliferator-activated receptor gamma (PPARγ), whose biological actions are to regulate glucose and lipid metabolism and suppress inflammatory gene expression) have shown a significant improvement in memory and cognition in AD patients (Landreth et al., 2008). The currently used multi-target-directed ligand (MTDLs), usually including a mix of inhibitors of acetyl-choline esterase (AChE) and of mono-amine oxidase (MAO) (Cavalli et al., 2008) may be complemented with ligands (NSAIDs, PPARγ agonists) that improve metabolic functions of neurons and microglia, in order to delay of prevent deterioration of AD.

CHAPTER 7

LPYFDa neutralizes Aβ-induced memory impairment and toxicity

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ABSTRACT

Misfolding, oligomerization and aggregation of the amyloid-beta (Aβ) peptide is widely recognized as a central event in the pathogenesis of Alzheimer's disease (AD). Recent studies have identified soluble Aβ oligomers as the main pathogenic agents and provided evidence that such oligomeric Aβ aggregates are neurotoxic, disrupt synaptic plasticity and inhibit long-term potentiation (LTP). A promising therapeutic strategy in the battle against AD is the application of short synthetic peptides which are designed to bind to specific Aβ-regions thereby neutralizing or interfering with the devastating properties of oligomeric Aβ species. In the present study we investigated the neuroprotective properties of the amyloid sequence derived pentapeptide LPYFDa *in vitro* as well as its memory preserving capacity against $A\beta_{42}$-induced learning deficits *in vivo*. In vitro we could show that neurons in culture treated with LPYFDa are protected against $A\beta_{42}$-induced cell death. Moreover, in vivo LPYFDa prevented memory impairment tested in a contextual fear conditioning paradigm in mice after bilateral intrahippocampal $A\beta_{42}$ injections. We thus showed for the first time that an anti-amyloid peptide like LPYFDa can preserve memory by reverting $A\beta_{42}$ oligomer induced learning deficits.

7.1 INTRODUCTION

Progressive neurodegeneration and cognitive decline are typical features of Alzheimer's disease (AD), the most common form of dementia (Haass and Selkoe, 2007). Besides the formation of neurofibrillary tangles, it is widely acknowledged that the aggregation of amyloid β (Aβ) initiates a complex series of events that ultimately results in neuronal cell death particularly in forebrain regions, which is paralleled by the cognitive and behavioral decline that is characteristic for pathogenesis of AD. Aβ is generated by sequential proteolytic cleavage from the amyloid-β precursor protein (APP) by beta-, and gamma-secretases (amyloidogenic processing). Alternatively APP can be cleaved by α-secretase within the Aβ sequence which prevents the generation of Aβ peptide. Once released, Aβ due to its physico-chemical properties has the strong tendency to misfold, oligomerize and to aggregate into fibrils and plaques (Wisniewski et al., 1997).

Although amyloid plaques represent a major hallmark of AD, they correlate poorly with the progression of the disease (Forman et al., 2007). Interestingly, more recent studies have identified soluble Aβ–oligomer assemblies as the main pathogenic agents which, in contrast to plaques, do correlate well with the mental decline observed in AD patients (Haass and Selkoe, 2007; Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000; Wang et al., 1999). Furthermore, soluble Aβ oligomers have been shown to be neuro- and synaptotoxic, and to inhibit long-term potentiation (LTP) (Chang et al., 2006; Chapman et al., 1999; Shankar et al., 2008; Walsh and Selkoe, 2007; Wasling et al., 2009). Acute application of oligomeric Aβ leads to internalization of glutamatergic AMPA and NMDA receptors and finally to synaptic downscaling (Hsieh et al., 2006; Shankar et al., 2007). However, it should be appreciated that next to its pathological properties, Aβ notably in very low physiological concentrations, may have important roles in synaptic plasticity and normal brain functioning (Pearson and Peers, 2006; Puzzo et al., 2008; Wasling et al., 2009). The endogenous level of Aβ in the brain is regulated by synaptic activity *in vivo*, suggesting a dynamic feedback loop involving APP metabolism and Aβ that may modulate synaptic activity (Haass and Selkoe, 2007). Recently, Garcia-Osta and coworkers showed that depletion of endogenous Aβ by a single intrahippocampal (i.h.) administration of anti- Aβ -antibody leads to disrupted memory retention in rats (Garcia-Osta and Alberini, 2009). During the pathogenesis of AD the equilibrium of Aβ generation and Aβ clearance is disturbed, which eventually leads to elevated Aβ levels, increased Aβ aggregation and impaired memory function (Wasling et al., 2009).

Multiple therapeutic strategies have been developed since the second half of the previous century (Harkany et al., 2000). Unfortunately, most of the currently available therapies target only the symptoms since acting on presumed downstream neurotoxic pathogenic mechanisms without tackling the cause and thus hardly able to affect the progression of the disease. Experimental data from animal studies

using immunotherapy in order to remove Aβ- from the brain were highly promising (Schenk et al., 1999). However, clinical trials with active immunization against Aβ were halted due to severe brain inflammation and premature death of several patients (Hock et al., 2003; Orgogozo et al., 2003).
Consequently, as a therapeutic strategy compounds were developed that could inhibit or delay the development of Aβ aggregation, fibrillization, and/or plaque formation and were thus potentially capable of protecting neurons from Aβ toxicity. As part of this approach small peptides derived from the Aβ sequence were designed such as the pentapeptide LPYFDa which seemed to offer a promising starting point to develop potential drugs that can somehow revert the devastating impact of Aβ aggregates. The advantage of such compounds, in comparison to other putative therapeutic approaches for AD such as vaccination, is that they specifically target the abnormal conformation of Aβ without interfering with any possible physiological function of the soluble, monomeric Aβ peptide.
There have been numerous attempts to develop treatments developed to interfere with various key steps in the amyloidogenic process. Promising putative treatments may be those designed to inhibit steps that precede Aβ peptide aggregation, by blocking production of the toxic soluble Aβ oligomers in the first place, or by reversing, somehow, the toxic effect of these oligomers.
In the present study we established an *in vivo* model in the mouse for Aβ induced memory impairment through a single bilateral injection of oligomeric $A\beta_{42}$ into the hippocampus and explored in this model whether LPYFDa can be beneficial against the Aβ-induced cognitive deficits. Furthermore, we investigated the neuroprotective properties of Aβ-derived synthetic beta-sheet breaker peptide Leu-Pro-Tyr-Phe-Asp-amide (LPYFDa) (Datki et al., 2004; Juhasz et al., 2009; Szegedi et al., 2005) against the neurotoxic effects of soluble $A\beta_{42}$ oligomers in cultured mouse primary cortical neurons (PCN). Finally, we employed molecular modeling and docking experiments to reveal part of the putative mechanism of action of these peptides.

7.2 MATERIAL AND METHODS

7.2.1 Compounds

The beta-sheet breaker LPYFDa was synthesized in our laboratories by a solid-phase procedure involving the use of Merrifeld resin and Boc chemistry. Purity control and structure verification were carried out by amino acid analysis and mass spectrometry as previously described (Zarandi et al., 2007). The control peptide (scrP), which is a scrambled version of the LPYFD peptide (Pro-Asp-Tyr-Leu-Phe-amide), and Aβ1−42 ($A\beta_{42}$) were purchased from EZBiolab Inc. (Carmel, USA). Anti-Aβ antibody (6E10) was obtained from Covance (Emeryville, USA). Other compounds used in this study were purchased from Invitrogen (Carlsbad, USA) or Sigma-Aldrich Corporation (St. Louis, USA).

7.2.2 Preparation of Aβ-oligomers

Oligomeric $A\beta_{42}$ was prepared as was described by Dahlgren and colleagues (Dahlgren et al., 2002). In short, the synthetic $A\beta_{42}$ peptide was initially dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to a concentration of 1 mM. The peptide solution was divided into aliquots and the HFIP removed by evaporation under vacuum (SpeedVac; Savant Instruments, Hyderabad, India). The dry peptide films were stored at -20°C until further processing. Before use, the dry film $A\beta_{42}$ was dissolved in anhydrous DMSO to 5 mM followed by bath sonication (Decon, Hove Sussex, UK) for 10 min, subsequently diluted in neurobasal medium to a final concentration of 100 μM (stock solution) and incubated at 4°C for 24 h to enable $A\beta_{42}$ oligomerization. The aggregation state and the secondary structure of the oligomeric $A\beta_{42}$ preparation were examined by sodium dodecyl sulfate (SDS) - PAGE Western blotting and Circular Dichroism (CD) spectrometry (Figure 7.1).

7.2.3 Gel electrophoresis and Western Blot Analysis

Gel electrophoresis and Western blot analysis were adopted from Stine et al., 2003 (Stine et al., 2003). Briefly, unheated samples in SDS sample buffer were applied to 10-20% tris-tricine gradient gels (Bio-Rad, Munich, Germany), electrophoresed using tricine running buffer and subsequently transferred to 0.45-μm polyvinylidene difluoride membranes (Millipore, Bilerca, USA). Membranes were blocked with 1% I-Block (Tropix, Bedford, USA) in Trisbuffered saline containing 0.0625% Tween-20. Blots were incubated with primary antibody 6E10 over night (1:2000; mouse monoclonal against Aβ residues 1–16; Covance Emeryville, USA). Immunoreactivity was detected using enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA) and imaged on an Kodak X-Ray film (Eastman Kodak Company, Rochester, USA) (Figure 7.1A). Molecular weight values were estimated using the PageRuler (Fermentas International, Ontario, Canada) pre-stained molecular weight marker.

7.2.4 CD spectrometry

CD measurements were performed at 25 °C on a CD Spectrometer Model 62DS (AVIV Associates Inc., Lakewood, USA) using a quartz cell of 0.1-cm pathlength. All spectra were averages of four scans; the resolution was 1 nm. The oligomerized Aβ was diluted in PBS to a concentration of 25 μM. The samples were sonicated for 10 min immediately after dissolution. CD spectra were expressed as mean residue ellipticity $[\Theta]_{MR}$ in units of deg cm^2 $dmol^{-1}$, (Figure 7.1B). The percentages of secondary structures were analyzed using the K2D2 program (Perez-Iratxeta and Andrade-Navarro, 2008).

7.2.5 Molecular Modeling

7.2.5.1 Stochastic Conformational Search. EDMC calculations

The conformational space was explored using the method previously employed by Liwo et al. (Liwo et al., 1996) which includes the electrostatically driven Monte Carlo (EDMC) method (Ripoll and Scheraga, 1988, 1990) implemented in the ECEPPAK (Letoha et al., 2005) package. Conformational energy was evaluated using the ECEPP/3 force field (Nemethy G, 1992). Hydration energy was evaluated using a hydration-shell model with a solvent sphere radius of 1.4 Å and atomic hydration parameters that have been optimized using nonpeptide data (SRFOPT) (Vila et al., 1991; Williams et al., 1992). In order to explore the conformational space extensively, we carried out 10 different runs, each of them with a different random number. Therefore, we collected a total of 5000 accepted conformations. Each EDMC run was terminated after 500 energy-minimized conformations had been accepted. The parameters controlling the runs were the following: a temperature of 298.15 K was used for the simulations. A temperature jump of 1,000 K was used; the maximum number of allowed repetitions of the same minimum was 50. The maximum number of electrostatically predicted conformations per iteration was 400; the maximum number of random-generated conformations per iteration was 100; the fraction of random/electrostatically predicted conformations was 0.30. The maximum number of steps at one increased temperature was 20; and the maximum number of rejected conformations until a temperature jump is executed was 100. Only *trans* peptide bonds ($\Theta \cong 180°$) were considered. All accepted conformations were then clustered into families using the program ANALYZE (Meadows et al., 1994; Pohorille and Pratt, 1990) by applying the minimal-tree clustering algorithm for separation, using all heavy atoms, energy threshold of 30 kcal.mol^{-1}, and RMSD of 0.75 Å as separation criteria. This clustering step allows a substantial reduction of the number of conformations and the elimination of repetitions. A more detailed description of the procedure used here is given in section *4.4 Computational Methods* of reference (Masman et al., 2009b).

7.2.5.2 Docking Studies

Two models for Aβ were used as target systems; the monomeric $A\beta_{42}$ elucidated by Crescenzi et al (monomeric model) (Crescenzi et al., 2002), PDB code 1IYT, and the pentameric aggregate $A\beta_{42}$ developed by Masman et al (pentameric model) (Masman et al., 2009a). The structures were prepared for docking study as follows: for the $A\beta_{42}$ molecules, water molecules were removed from the PDB file and hydrogen atoms were added; Gasteiger charges, atomic solvation parameters and fragmental volumes were merged to the target system. For both LPYFDa and scrP, the structure of the most populated family (results from the *EDMC calculations*) was taken as initial conformation. Gasteiger charges were assigned and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking.

The docking energy grid was produced with the auxiliary program AutoGrid. The grid dimensions were 60x60x60 for the monomeric model, and 90x60x60 for the pentameric model, points along the x-, y- and z-axes, with points separated by 0.375Å. The grids were chosen to be sufficiently large to cover significant portions of the putative binding sites. The center of the pentapeptide was positioned at the grid center. All graphic manipulations and visualizations were performed by means of the AutoDock Tools (Sanner, 1999) and the Chimera (Pettersen et al., 2004) programs, and ligand docking with AUTODOCK 4 (Morris et al., 2009). The Lamarckian genetic algorithm was utilized and the energy evaluations were set at $2.5x10^6$. A total of 250 accepted conformations were collected. Other parameters were set to default values.

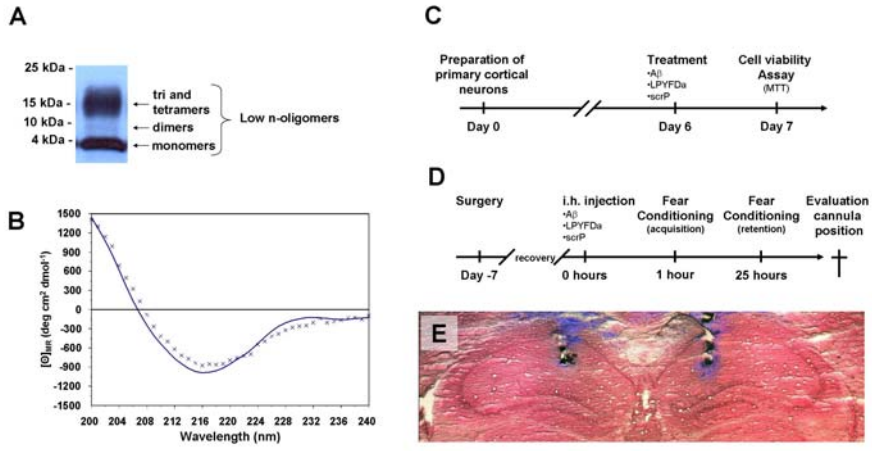

Figure 7.1 *A) Oligomeric preparation of $A\beta_{42}$ was examined by Western blot using the anti-Aβ antibody 6E10 (1:2000). Bands correspond to monomeric up to tetrameric forms of $A\beta_{42}$. B) CD spectrum of (x) $A\beta_{42}$ in PBS after 24 hours incubation at 4 °C.(—) fitted line data using K2D2 program. C) Schematic outline of the experimental procedure in vitro. PCN from C57BL6 mouse embryos (E14) were cultured for 6 days and then treated for 24 h with different concentrations of oligomerized $A\beta_{42}$, beta-sheet breaker peptides or combinations of both. On day 7 medium was completely exchanged and 24 h later the cell viability was assessed by an MTT reduction assay. D) Schematic outline of the experimental setup in vivo. C57BL/6J mice were cannulated 7 days prior i.h. injection with oligomerized $A\beta_{42}$ and/or beta-sheet breaker peptides. One hour after the injection the animals were trained in a contextual fear conditioning paradigm. 24 h after the training session memory performance was assessed. Cannula position was evaluated by methylene blue injection after the behavioural test. E) Representative coronal brain sections of a bilateral dorsal hippocampal (i.h.) injection with methylene blue counterstained with nuclear fast red.*

7.2.6 Primary cortical neuron culture

Primary cortical neurons were prepared from embryonic brains (E14) of C57Bl/6J mice. The cortices were carefully dissected, meninges were removed and the neurons separated by trituration. Cells were plated on poly-D-lysine pre-coated plates at a density of 1.2 x 10^5 cells/well (96 well plates). Neurobasal medium supplemented with 2% (v/v) B27-supplement, 0.5 mM glutamine and 1% (v/v) penicillin/ streptomycin was used as a culture medium. After 48 h neurons were treated with 10 µM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and after 6 days of *in vitro* culture, the neurons were used for experiments.

7.2.7 Treatment of cells

Possible toxicity of LPYFDa, its control peptide scrP and $A\beta_{42}$ oligomers was determined by incubating neuronal cultures for 24 h with different concentrations of the peptide solutions. The neuroprotective effect of LPYFDa was assessed by incubating neurons (cultured in 96 well plates) for 24 h with 20 µM oligomeric Aβ in the presence or absence of different concentrations of LPYFDa or control peptide. After the treatment, the medium was completely exchanged, and 24 h later, the cell viability was determined by an MTT-assay (Figure 7.1C). All treatments were performed in triplicates and the experiments were repeated at least two times.

7.2.8 Determination of cell viability by MTT-assay

Neuronal viability was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide] assay as described previously (Mosmann, 1983). 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2 h of incubation, cells were lysed in acidic propan-2-ol solution (37% HCl/ propan-2-ol: 1/166). The absorbance of each well was measured with an automated ELISA plate reader (Bio-Rad, Munich, Germany) at 595 nm with a reference filter at 630 nm.

7.2.9 Animals

Behavioral experiments were performed with 9–12 weeks old male C57Bl/6J mice (Harlan, Horst, The Netherlands). Individually housed mice were maintained on a 12 h light/dark cycle (lights on at 7.00 a.m.) with food (Hopefarms, standard rodent pellets) and water ad libitum. A layer of sawdust served as bedding. The animals were allowed to adapt to the housing conditions for 1–2 weeks before the experiments started. The procedures concerning animal care and treatment were in accordance with the regulations of the Ethical Committee for the use of experimental animals of the University of Groningen (DEC4668C).

7.2.10 Animal surgery

Double guide cannulae type C235 (Plastics One, Roanoke, USA) were implanted in the brain using a Kopf stereotactic instrument during Hypnorm/Midazolam (10 ml/kg, i.p.) anesthesia under aseptic conditions as previously described (Nijholt et al., 2008) with anteroposterior (AP) coordinates zeroed at Bregma directed toward both dorsal hippocampi (i.h.), AP -1.5 mm, lateral 1 mm, depth 2 mm (Franklin and Paxinos, 1997). Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull with dental cement (3M ESPE AG, Seefeld, Germany). Administration of 1 mg/ml finadyne (2.5 mg/kg s.c.) before the surgery served as analgesic. The animals were allowed to recover for 6–7 days before the behavioral measurements started.

7.2.11 Intrahippocampal injections

Bilateral i.h. injections were performed under short isofluran anesthesia using a Hamilton microsyringe fitted to a syringe pump unit (TSE systems, Bad Homburg, Germany) at a constant rate of 0.3 µl/min (final volume: 0.3 µl per side). The amount of injected $A\beta_{42}$ was of 15, 30 or 60 pmol and LPYFDa as well as the control peptide scrP in an amount of 150 pmol into the dorsal hippocampus. PBS (pH 7.5) served as vehicle. One hour after the injection the animals were subjected to a training session in a fear conditioning paradigm (Figure 7.1D). The number of animals per group varied from 5 to 9.

7.2.12 Fear conditioning

Fear conditioning was performed in a plexiglas cage (44 x 22 x 44 cm) with constant illumination (12 V, 10W halogen lamp, 100–500 lux). The training (conditioning) consisted of a single trial. Before each individual mouse entered the box, the box was cleaned with 70% ethanol. The mouse was exposed to the conditioning context for 180 s followed by a scrambled footshock (0.7 mA, 2 s, constant current) delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 s after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 h after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 s without footshock presentation. Freezing, defined as the lack of movement except for respiration and heart beat, was assessed as the behavioral parameter of the defensive reaction of mice by a time-sampling procedure every 10 s throughout memory tests. In addition, mean activity of the animal during the training and retention test was measured with the Ethovision system (Noldus, Wageningen, The Netherlands).

7.2.13 Histology

Immediately after the behavioral test mice were injected i.h. with methylene blue solution during sodium-pentobarbital anesthesia (0.1 ml/ 10g, i.p.). Brains were removed and serially sectioned at 50 µm. Sections were stained on glass for 5 min in 0.1% nuclear fast red solution. To identify the location of the injection, sections were analyzed using light microscopy. Only data from animals in which the proper intrahippocampal site of injection was confirmed, were evaluated (Figure 7.1E).

7.2.14 Statistical analysis

Behavioral data were analyzed by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test to determine statistical significance. For statistical analysis of the MTT assays, an unpaired Student's *t* test with unequal variance was used. A p-value < 0.05 was considered to be statistically significant. Data are presented as mean value ± standard error of the mean (SEM).

7.3 RESULTS

7.3.1 Characterization of the oligomeric $A\beta_{42}$

The state of aggregation of the oligomeric $A\beta_{42}$ preparation was determined by Western blot analysis. The results confirmed that the $A\beta_{42}$ preparation consisted of a mixture of small molecular weight $A\beta_{42}$ oligomers from monomeric to tetrameric $A\beta_{42}$ (Figure 7.1A). In addition we used CD spectrometry to characterize the secondary structure of $A\beta_{42}$ in solution. We could show that our oligomeric $A\beta_{42}$ preparation consists of 43.02% β-sheets, 5.40% α-helix and 51.58% random-coil conformation (Figure 7.1B), which shows a good correlation to the conformation behavior of $A\beta_{42}$ aggregates observed by Masman et al (Masman et al., 2009a).

7.3.2 Molecular modeling and Stochastic Conformational Search. EDMC calculations

To have a better view at the molecular lever, it is crucial to assess the conformational behavior of the pentapeptides in solution. Therefore, LPYFDa and scrP were selected for energy calculations to determine the biologically relevant conformations. The results of the theoretical calculations are summarized in Table 7.1.

Calculations yielded a large set of conformational families for each peptide studied. The total number of conformations generated was 62515 and 65056, for LPYFDa and scrP respectively, whereof 5000 conformations for each pentapeptide were accepted. In the clustering procedure, an R.M.S.D (Root Mean Square Deviation) of 0.75 Å and a ΔE of 30 kcal mol^{-1} were used. The number of families after clustering was 220 and 323, for LPYFDa and scrP, resp. The total number of families accepted with a relative population higher than 0.50% was 10 and 25, for LPYFDa and scrP, resp. that sum up to *ca* 90% of all conformations. All low-energy conformers of pentapeptides studied here were then compared to each other. The comparison involved the spatial arrangements, relative energy and populations. The LPYFDa evaluation showed that the most populated family (50.04%) is also the energetically preferred one, while its second most populated family (15.36%) has a relative energy of 0.03 kcal mol^{-1} above the global minimum. This small energetic difference is due to a slight reorientation of the side-chain of the residue Phe (see Figure 7.2A), while the rest of the structure showed approximately the same orientation. On the other hand, the most populated family of scrP (22.92%) showed a relative energy of 1.18 kcal mol^{-1} above the global minimum and a relative population of 6.38%. It was observed that LPYFDa is confrontationally, generally more restricted than scrP, with a preference to form folded structures, while scrP tended to form semi-extended or fully-extended conformations. Spatial views of selected conformations, for LPYFDa and scrP are shown in Figure 7.2.

***Table 7.1**: Conformational search and clustering results for LPYFDa and scrP optimized at the EDMC/SRFOPT/ECCEP/3 level of theory. Total (**E_{tot}**, kcal mol^{-1}) and relative (**ΔE**, kcal mol^{-1}) energies are also shown. All conformational families shown here have relative population (%PF) higher than 0.5%.*

LPYFDa				
	Electrostatic	Random	Thermal	Total
Generated [a]	4233	58242	40	62515
Accepted [b]	535	4431	34	5000
Family	**NF [c]**	**%PF [d]**	**E_{tot}**	**ΔE**
1	2502	50.04	-70.79	0.00
2	768	15.36	-70.76	0.03
3	370	7.40	-69.43	1.36
4	262	5.24	-70.12	0.66
5	261	5.22	-69.31	1.47
6	247	4.94	-70.25	0.54
7	62	1.24	-69.37	1.42
8	47	0.94	-70.13	0.66
9	35	0.70	-69.75	1.03
10	30	0.60	-68.45	2.34
scrP				
	Electrostatic	Random	Thermal	Total
Generated [a]	4288	60708	60	65056
Accepted [b]	362	4595	43	5000

Family	NF [c]	%PF [d]	E_{tot}	ΔE
1	1146	22.92	-49.71	1.18
2	635	12.70	-50.16	0.73
3	478	9.56	-49.95	0.93
4	329	6.58	-50.88	0.00
5	272	5.44	-49.66	1.22
6	250	5.00	-49.94	0.94
7	190	3.80	-50.19	0.69
8	116	2.32	-49.71	1.17
9	114	2.28	-50.57	0.31
10	101	2.02	-49.44	1.44
11	97	1.94	-49.63	1.26
12	88	1.76	-50.23	0.65
13	60	1.20	-50.25	0.64
14	53	1.06	-49.47	1.41
15	51	1.02	-49.81	1.07
16	48	0.96	-49.39	1.50
17	45	0.90	-48.58	2.30
18	39	0.78	-48.78	2.10
19	39	0.78	-48.22	2.66
20	38	0.76	-48.97	1.91
21	37	0.74	-49.23	1.65
22	32	0.64	-49.74	1.14
23	30	0.60	-48.90	1.98
24	27	0.54	-49.03	1.86
25	26	0.52	-49.07	1.81

[a] Number of conformations generated electrostatically, randomly and thermally during the conformational search.
[b] Number of conformations accepted from those generated electrostatically, randomly and thermally during the conformational search.
[c] *NF* represents the total number of conformational families as result of the clustering run.
[d] *%PF* represents the percent relative population based on a total of 5000 accepted conformations.

7.3.3 Docking Studies

7.3.3.1 Monomeric Model

Two potential binding sites were found by using a single blind docking run (results not shown) on the monomeric $A\beta_{42}$ molecule (PDB code 1IYT), which comprises two α-helix moieties (residues 8-25 and 29-39) connected by a loop (residues 26-28). Site I encompasses the residues 21-26 containing Glu^{22} and Asp^{23}, which were previously identified as residues for aggregation of the oligomers (Buchete and Hummer, 2007; Masman et al., 2009a). Site II includes residues 6-12 located in the portion of the molecule that loses all structural organization after oligomer formation, thus forming the so-called disordered region. In table 7.2 the two most populated families of the complexes of LPYFDa and scrP with the monomeric $A\beta_{42}$ are summarized. LPYFDa showed lower binding energies, while site II was in general energetically preferred over site I but families on site II poorly populated. In general it can be concluded that LPYFDa binds stronger to the monomeric $A\beta_{42}$ than scrP.

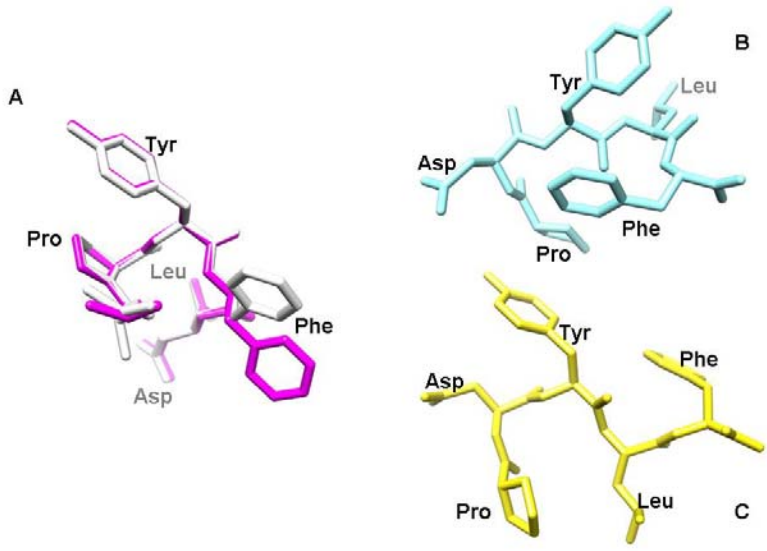

Figure 7.2. *Stereoview of selected conformations for LPYFDa and scrP optimized at EDMC/SRFOPT/ECCEP/3 level of theory. (A) overlapping of the two most populated and energetically preferred families of LPYFDa, family 1 (ΔE = 0.00 cal.mol^{-1}, white) and family 2 (ΔE = 0.03 kcal.mol^{-1}, pink) . (B) Family 4 with a relative energy (ΔE) of 0.00 kcal.mol^{-1} and (C) Family 1 with an ΔE = 1.18 kcal.mol^{-1} of ScrP. All hydrogen atoms have been deleted for more clarity.*

7.3.3.2 Pentameric Model

As previously reported by Masman et al. (Masman et al., 2009a), the $A\beta_{42}$ aggregates contain two β-sheet moieties (β1, residues 18-26 and β2, residues 31-42) organized into a parallel interchain orientation, which were proposed as intermolecular binding sites for the pentameric conformation. Moreover, a third possible site for interactions with ligand molecules was postulated, which involves the β1 and β2 portions of the chain located at the edge of the aggregate. This site is orientated into axis of the oligomers, in the same direction where the oligomers grows by aggregation.

Table 7.2: *The two most populated families of LPYFDa and scrP found by docking simulations on the monomeric and pentameric Aβ_{42} peptide and the corresponding binding energies (E_B) of the complexes. The binding constant (K_B) and the relative populations (%P) are also shown.*

			LPYFDa			scrP		
			E_B (kcal mol^{-1})	K_B[a] (M)	%P	E_B (kcal mol^{-1})	K_B[a] (M)	%P
Monomeric Aβ_{42}	Site I	1	-4.42	5.78x10^{-4}	13.60	-3.67	2.05x10^{-3}	4.80
		2	-4.35	6.49x10^{-4}	8.00	-3.18	4.66x10^{-3}	4.00
	Site II	1	-4.95	2.35x10^{-4}	6.40	-4.10	9.89x10^{-4}	3.20
		2	-4.66	3.85x10^{-4}	3.20	-3.96	1.24x10^{-3}	2.00
Pentameric Aβ_{42}	TOP	1	-5.67	6.99x10^{-5}	5.60	-4.50	5.03x10^{-4}	5.20
		2	-5.04	2.02x10^{-4}	3.60	-4.73	3.39x10^{-4}	3.60
	β1	1	-8.19	9.88x10^{-7}	8.80	-6.17	3.00x10^{-5}	5.20
		2	-7.05	6.75x10^{-6}	6.80	-5.01	2.11x10^{-4}	4.40
	β2	1	-4.75	3.32x10^{-4}	6.00	-4.68	3.69x10^{-4}	2.80
		2	-4.77	3.20x10^{-4}	3.20	-4.92	2.48x10^{-4}	2.40

[a] K_B is calculated in with the equation K_B=exp((ΔG*1000.)/(Rcal*TK), where ΔG is the docking energy, Rcal is 1.98719 and TK is 298.15

Table 7.2 shows the two most populated families of the complexes of LPYFDa and scrP with the pentameric Aβ_{42}. For all the sites proposed, LPYFDa showed lower binding energies than scrP. Interestingly, both pentapeptide LPYFDa and scrP, revealed an energetic preference for site β1. LPYFDa was designed on the basis of Soto's pentapeptide LPFFD, which derives from the amino acid sequence Leu^{17}-Val-Phe-Phe-Ala^{21} of Aβ_{42}, being the β1 portion of the aggregate. Figure 7.3 shows the atomic details of the interactions of the best complex (family β1 1, in table 7.2) found between LPYFDa and the pentameric Aβ_{42}. It can be appreciated that the N-terminal of LPYFDa formed a double salt bridge with the Glu^{22}'s of the second and third chain of the aggregate. Also, a second salt bridge links the Lys16 to the Asp residue of LPYFDa. All salt bridges, indicated with a block-arrow that points to the positive member of the interaction, revealed an interacting distance of approximately 3.5 Å. All the ligand-target contacts are depicted as wireframe spheres. An important hydrophobic pocket was formed between the residues Phe^{20}

and Val[18] of the aggregate, and the residues Leu, Pro and Phe of the pentapeptide LPYFDa. This hydrophobic pocket is indicated with a dashed line.

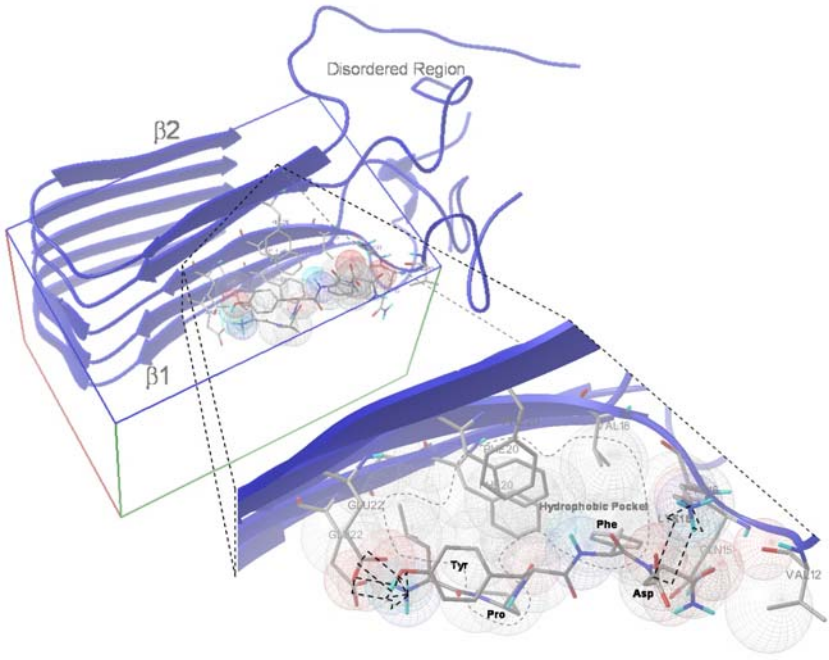

Figure 7.3. *A steric view of binding between the pentameric Aβ_{42} model and LPYFDa. Salt bridges are signalized with block-arrows. All the ligand-target contacts are depicted as wireframe spheres.*

7.3.4 LPYFDa is neuroprotective against oligomeric Aβ_{42} in vitro

Part of our study was to assess the neuroprotective potential of the beta-sheet breaker LPYFDa against Aβ-induced toxicity. Therefore, we first determined the toxic effect of the Aβ_{42} oligomer preparation in vitro on cultured primary cortical neurons (PCN). Neuronal cultures were exposed to increasing concentrations of oligomerized Aβ_{42} for 24 h, followed by an MTT-reduction assay to assess cell viability. The results showed a clear Aβ-induced, dose dependent decrease in cell survival reaching significance at concentrations higher than 10 μM (Figure 7.4A). For the subsequent experiments we used oligomeric Aβ_{42} at a concentration of 25 μM as a toxic stimulus.

Second, we investigated if LPYFDa or the control peptide scrP exhibited any toxicity to PCN and whether the beta-sheet breaker peptides were capable to overcome the toxic effect of oligomeric Aβ_{42}. For this purpose PCN were exposed to different concentrations of LPYFDa or scrP alone, Aβ_{42} alone (25 μM) or beta-sheet breaker peptides and Aβ_{42} together for 24 h. The results showed that LPYFDa and scrP alone were not toxic to PCN at any tested concentration (Figure 7.4B).

Furthermore, the control peptide scrP was not able to protect the neurons from Aβ-induced toxicity (Figure 7.4C). In contrast, we could demonstrate a dose dependent neuroprotective effect of LPYFDa against Aβ-induced toxicity, reaching significance at 4 µM LPYFDa and higher (Figure 7.4D).

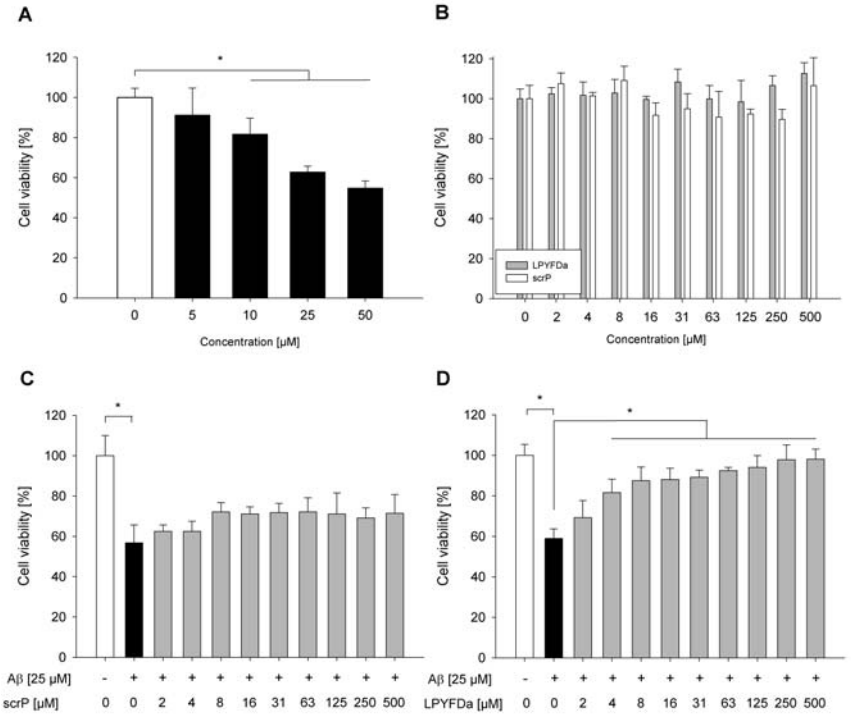

Figure 7.4. *Cell viability of PCN as determined by an MTT-assay. Neurons treated with increasing concentrations of A) $A\beta_{42}$ or B) pentapeptides LPYFDa and scrP incubated for 24 h. Neuroprotection by C) scrP or D) LPYFDa was determined by co incubating increasing concentrations of the pentapeptides with 25 µM $A\beta_{42}$ for 24 h. Bars indicate the mean cell viability in % relative to untreated controls ± SEM. (* = significant at $p < 0.05$).*

7.3.5 A single intrahippocampal injection of oligomeric $A\beta_{42}$ induces cognitive deficits in contextual fear conditioning

One hour prior to the training session in a contextual fear conditioning paradigm C57BL/6J mice received a single injection of oligomeric $A\beta_{42}$ (15, 30 or 60 pmol) or vehicle. Injections did not affect locomotion or the shock reaction during training (data not shown). The vehicle injected animals displayed an average relative freezing behavior of 58.9± 2.9% which did not differ from untreated control animals (61.1± 2.4 %). The mice injected with $A\beta_{42}$ showed a dose dependent decrease in freezing behavior (Figure 7.5A). 15 pmol $A\beta_{42}$ caused an average relative

freezing of 50.0±4.2 % which was not significantly different from the vehicle-injected animals. However, the mice injected with 30 pmol and 60 pmol of $A\beta_{42}$ had significantly reduced freezing scores compared to the vehicle group (36.2± 4.6%; p=0.024 and 33.3± 5.3%; p=0.037).

Next, we investigated whether LPYFDa is able to revert the Aβ-induced memory deficits. Therefore, we injected mice with 150 pmol LPYFDa or 150 pmol of the non specific control peptide scrP in the presence or absence of 30 pmol oligomerized $A\beta_{42}$ into the hippocampus. The LPYFDa and the scrP injected mice showed an average freezing of 61.1± 5.0% and 63.0± 5.5% which did not significantly differ from the untreated (61.1± 2.4%) or vehicle injected group (58.9± 2.9%).

However, LPYFDa co-injected with $A\beta_{42}$ was able to abolish the $A\beta_{42}$ oligomer-induced memory impairment (57.4± 5.2% vs 36.2± 4.46%; p=0.039). Co-injection of Aβ with the control peptide scrP, which resulted in an average freezing score of 48.0± 4.9%, did not significantly reverse the Aβ-induced memory deficits (Figure 7.5B).

These results indicate that LPYFDa can reverse the detrimental effects of $A\beta_{42}$ oligomers and subsequent impaired memory performance.

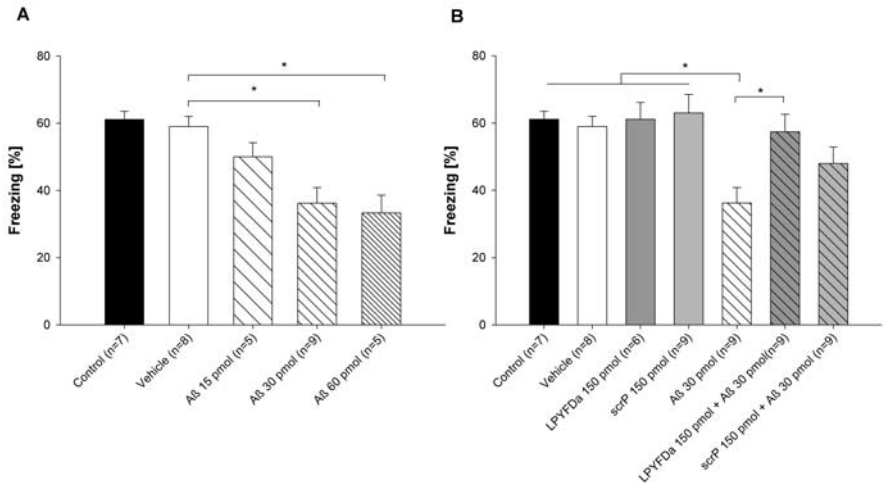

Figure 7.5. *Effect of $A\beta_{42}$ and LPYFDa on contextual fear conditioning. A) A single i.h. injection of oligomeric $A\beta_{42}$ led to a dose dependent significant decrease in conditioned fear when compared to a vehicle injection. B) Co-injection of $A\beta_{42}$ with LPYFDa prevented the Aβ-induced memory impairment significantly, whereas the non-specific control peptide scrP failed to revert memory deficits. Bars indicate the mean relative freezing score in % ± SEM. Differences were determined by ANOVA (* = significant at $p < 0.05$).*

7.4 DISCUSSION

In the present study we could show that $A\beta_{42}$ oligomers are toxic to primary cortical neurons in culture in a dose-dependent manner (Figure 7.4A). These results are in line with studies by Dahlgren and colleagues, who reported that the oligomeric form of $A\beta_{42}$ is 10 fold more toxic when compared to the fibrillar form (Dahlgren et al., 2002). These and our present findings support the growing general view that in particular oligomeric $A\beta_{42}$ peptides contribute to the progressive neuronal loss and the associated memory impairment observed in AD patients. Indeed, several studies showed that elevated levels of soluble oligomeric Aβ correlate strongly with cognitive decline in AD patients (McLean et al., 1999; Wang et al., 1999). Walsh et al. observed that a low-n oligomeric assembly of naturally secreted human Aβ alters hippocampal synaptic plasticity by inhibiting long-term potentiation (Walsh et al., 2002). Also the number of dendritic spines was dramatically decreased when neurons were incubated with Aβ oligomers, but not with monomers (Walsh et al., 2002). However, the loss in spines could be reverted by treating neurons with an anti-Aβ antibody (Shankar et al., 2007).

A possibility to counteract the injurious effects of oligomeric Aβ is by modulating its aggregation. Crucial for the aggregation process of the Aβ molecule are the hydrophobic residues (amino acids 17-21: LVFFA) within the internal region of the Aβ peptide. Experiments by Hilbich and colleagues revealed that replacement of those hydrophobic residues by hydrophilic residues results in impaired fibril formation (Hilbich et al., 1992). These and other findings eventually led to the concept of beta-sheet breakers as therapeutic strategy for AD as proposed by Soto et al. (Soto, 1999; Soto et al., 1996). The initially synthesized compounds were peptides of 11 to 5 amino acids targeting the center region of the Aβ peptide and evolved to compounds like LPFFD (iAβ5) and/or LPYFDa. These pentapeptides are partially homologous to this hydrophobic center region and bind with a relatively high affinity to Aβ (Hetenyi et al., 2002a; Permanne et al., 2002; van Groen et al., 2009) by similar intermolecular interactions, leading to a competitive replacement of Aβ molecules. A major drawback with peptide drugs for neurological disorders is their rapid degradation in vivo by proteolytic enzymes and their poor blood-brain permeability (Adessi and Soto, 2002). These issues were overcome by chemical modifications, like C-terminal amidation and N-terminal carboxylation, which resulted in increased half life *in vivo* and rapid brain up-take (Permanne et al., 2002). Although, our knowledge of the biochemistry in respect to catabolism and brain uptake of certain aggregation inhibitor peptides has developed greatly in recent years, the detailed mechanisms of action are still poorly understood. Therefore, it is essential to elucidate and study the three dimensional structure of the Aβ peptide/aggregation inhibiting peptide complex to gain more insight into the molecular dynamics of the Aβ aggregation process.

We used computational modeling and docking experiments to address this question. Interestingly our docking results showed that LPYFDa binds preferably to the β1 portion of the aggregate (Table 7.2 and Figure 7.3), which has a good correlation with the design of this pentapeptide, since LPYFDa derives from the

wild-type sequence Leu[17]-Val-Phe-Phe-Ala[21] of $A\beta_{42}$, which is contained in this portion of the aggregate. Our docking results did not show any binding preference of LPYFDa (nor scrP) for the monomeric or the pentameric Aβ.

In the present study we showed that the pentapeptide LPYFDa can protect cultured neurons from oligomeric Aβ-induced cell death. Datki et al. reported similar results on neuronal-like cell lines, e.g. SHSY-5Y cells. They could show that a 5-fold molar excess of LPYFDa protects these cells from toxic effects of fibrillar Aβ (Datki et al., 2004; Fulop et al., 2004). In our study we could confirm that a molar excess of LPYFDa can prevent $A\beta_{42}$ mediated neurotoxicity. Moreover, we demonstrated a dose-dependent protective effect of LPYFDa and that this pentapeptide already has significant neuroprotective properties even with a 6-fold molar excess of oligomeric $A\beta_{42}$. Our results are also in agreement with other studies reporting protection by LPYFDa against the rapid neuromodulatory action of fibrillar $A\beta_{42}$ demonstrated by *in vitro* and *in vivo* electrophysiology (Szegedi et al., 2005).

We found that a single bilateral intrahippocampal injection of oligomeric $A\beta_{42}$ impairs memory formation if applied 1 h before the training session in a contextual fear conditioning paradigm. These findings are in line with several other studies, which consistently report on memory deficits after intracerebral injections of $A\beta_{42}$ peptides, although the experimental conditions vary in terms of the injected peptide, injection procedure and behavioral tasks employed (Ammassari-Teule et al., 2002; Christensen et al., 2008; Harkany et al., 2001; Malin et al., 2001; Nakamura et al., 2001; O'Hare et al., 1999). It should be noted that these injections obviously lead to Aβ levels beyond the basal levels necessary for proper synaptic functioning. Garcia-Osta showed that neutralizing endogenous Aβ by an anti-Aβ antibody resulted in memory impairment which implies that physiological soluble Aβ levels are required for proper memory function (Garcia-Osta and Alberini, 2009), which was recently confirmed by a study of Morley et al (Morley et al., 2009)

The peptide LPFFD, which was designed by the Soto group was able to prevent fibrillogenesis in a rat brain model (Soto et al., 1998) and to reduce Aβ deposition a transgenic Alzheimer mouse model (Permanne et al., 2002). Most interestingly, iAβ5p was shown to reverse the memory impairment caused by intrahippocampal injections of Aβ fibrils in rats (Chacon et al., 2004). However, these studies did not reveal whether impairment of learning and memory after oligomeric Aβ injections could be counteracted by such compounds. A novelty of our study is that we used Aβ oligomers instead of the less effective Aβ fibrils. Therefore, an important aim of our study was to establish if a compound like LPYFDa is able to prevent Aβ oligomer–induced learning and memory deficits. We showed that a 5-fold molar excess of LPYFDa to Aβ could overcome the detrimental effects of Aβ oligomers on memory. Thus, we provide evidence that so-called beta-sheet breaker peptides such as LPYFDa bear therapeutic potential against Aβ-induced memory impairment. Moreover, it was recently demonstrated that intraperitoneally administered LPYFDa is able to cross the blood brain barrier and protects synapses against excitatory action of fibrillar Aβ (Juhasz et al., 2009).

The mechanism how these pentapeptides exert their protective effects on cell death and behavior is not yet fully understood. However, it should be noted that the two

actions of Aβ reported here, namely, the neuronal degeneration following application of Aβ oligomers *in vitro* and memory loss following injections of Aβ oligomers *in vivo* may be unrelated. In hippocampal tissue extracted one hour after Aβ injection we were unable to detect any caspase-3 cleavage which would indicate apoptotic cell death (data not shown). Furthermore, there are no studies so far that show toxic effects of oligomeric Aβ on neurons *in vivo*. Therefore, the effects on memory following an hour post-injection may not have a direct correlation with neuronal degeneration, but could simply reflect the effects of Aβ on synaptic integrity. It is well documented that the main effects of Aβ on synapses are inhibition of LTP (Chapman et al., 1999; Kamenetz et al., 2003) and elimination of postsynaptic glutamate receptors (Almeida et al., 2005; Chang et al., 2006; Ting et al., 2007). Remarkably, the Aβ induced synaptic dysfunction occurs rather rapidly, starting with 20 minutes after application, and does not need chronic exposure (Chen et al., 2000; Chen et al., 2002; Townsend et al., 2006; Wang et al., 2004). Furthermore, it is likely that the injected oligomeric Aβ does not remain unchanged in terms of its conformation, and the effects on memory may be due to changes of Aβ to other conformations. The importance of the Aβ conformation on memory was demonstrated by Lesne et al (Lesne et al., 2006), who showed that only specific Aβ protein assemblies in the brain are able to impair memory (Lesne et al., 2006).

It remains elusive whether aggregation inhibiting peptides like LPYFDa directly bind to Aβ, and thereby prevent possible interactions between Aβ and neuronal membrane proteins, and in this way neutralize its toxic effect. We could consider two options of interaction with Aβ, 1) the pentapeptides bind to the monomeric Aβ, thus preventing and/or retarding the formation of toxic oligomers, and 2) the pentapeptides bind the Aβ oligomers already formed preventing and/or modulating, somehow, its neurotoxic properties. On the other hand, both above mentioned possibilities might act simultaneously comprising a third possible way of action of this peptide toward its neuroprotective effects.

Being aware of the many possible biological pathways that these peptides might follow while inducing neuroprotection, it is interesting to see whether these peptides show a preference to bind the monomeric Aβ or its soluble oligomers. Previous studies using CD spectrometry and molecular docking studies have been carried out on LPYFDa and other so-called β-sheet breaker peptides (Datki et al., 2004; Hetenyi et al., 2002a; Hetenyi et al., 2002b; Laczko et al., 2008) but none of them so far have demonstrated proof at the molecular level of the β-sheet breaking properties of these peptides. We also might consider the possibility that these peptides might act as "glue" that promote elongation to biologically inert larger aggregates, or conversely, bind to the monomeric Aβ and this way inhibits oligomer formation. By either of these actions, or both, the neurotoxicity of Aβ is decreased or reverted.

In summary our findings provide evidence on how and where LPYFD interacts with Aβ mono- and oligomers, that LPYFDa neutralizes the neurotoxic activity of soluble Aβ oligomers, and in the present conditions can effectively prevent the oligomer-induced deficits in memory performance.

CHAPTER 8

Neuroprotective action and neutralization of $A\beta_{42}$-induced memory impairment by a novel *in silico* designed N-methyl amino acid containing peptide

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ABSTRACT

There is overwhelming evidence that points towards the central role of the Abeta-peptide in pathogenesis of Alzheimer's disease (AD). In particular the misfolding and aggregation of Aβ into soluble oligomeric assemblies is associated with synaptic toxicity and cognitive dysfunction. A promising therapeutic approach for AD treatment is to neutralize or interfere with the devastating properties of oligomeric Abeta species, which can be achieved by short synthetic peptides (e. g. LPYFDa) designed to bind to specific Abeta-regions.
In this study, we present a novel rationally designed peptide (PN22), employing the in silico approach (conformational analysis, molecular dynamics and docking studies). The design of PN22 is based on the conformational behavior of $A\beta_{42}$ aggregates.
Furthermore, we evaluated the therapeutic potential of the novel peptides by testing them for their neuroprotective properties against oligomeric $A\beta_{42}$ in vitro, as well as their memory preserving capacity in vivo. We have been able to show that neurons in culture treated with PN22 are protected against $A\beta_{42}$ -induced cell death. Moreover, PN22 prevented memory impairment tested in a contextual fear conditioning paradigm in mice.
In conclusion we can demonstrate that the peptide PN22 can neutralize the neurotoxic effects of soluble $A\beta_{42}$ oligomers in vitro and in vivo. In the latter condition we obtained proof of principle that short synthetic peptides can effectively prevent the $A\beta_{42}$ oligomer-induced deficits in memory performance.

8.1 INTRODUCTION

Alzheimer's disease (AD) is a complex multifactorial neurodegenerative syndrome characterized by the patient's memory loss and impairment of a wide range of cognitive abilities. This devastating disease affects more than 20 million people worldwide and, as a consequence of the worlds aging population, the prevalence of AD is expected to increase to endemic dimensions both in developed and developing nations (Blennow et al. 2006; Pratico and Delanty 2000). AD is the most extensively studied amyloid-based disease, whose main hallmarks are characterized by pathological high levels of amyloid deposits in frontal brain regions (senile or amyloid or neuritic plaques) and neurofibrillary tangles (Haass and Selkoe 2007; Holtzman and Mobley 1991). The major components of senile plaques are small peptides of 39-43 amino acids called β-amyloid (Aβ) which are produced through endoproteolysis of the amyloid precursor protein (APP). During the pathogenesis of AD the equilibrium of Aβ production and Aβ clearance is disturbed, which eventually leads to elevated Aβ levels, increased Aβ aggregation and consequently impaired memory function (Wasling et al. 2009). Recent evidence now indicates that particularly soluble oligomeric forms of $A\beta_{42}$ are to be considered as the most toxic form of this peptide, and correlate well with the progression and cognitive decline of the disease (Lesne et al. 2006; Wasling et al. 2009)

Due to the high incidence that AD has in the human aging population, there is an evident need of therapeutic agents that could at least significantly delay the course of the disease (Hardy and Selkoe 2002; Wolfe 2002). Several research strategies currently attempt to develop therapies and therapeutic agents that aim to reduce Aβ production, enhancing its clearance and/or preventing or retarding the amyloidogenic processes. Amongst them, the use of peptides or peptidomimetic molecules derived from the Aβ sequence is particularly appealing (Granic et al. 2010; Soto et al. 1998; Tjernberg et al. 1996a; Wolfe 2002). Promising putative treatments may be those designed to inhibit steps that *precede* Aβ peptide aggregation, by blocking the production of toxic soluble Aβ oligomers in the first place, or by reversing, somehow, the toxic effect of these soluble oligomers (FIGURE 8.1).

In fact, some short peptides that are derived from the Aβ sequence have already been reported to specifically interact with Aβ and cause interferences with its neurotoxic effects. LPFFD (Soto et al. 1996; Soto et al. 1998), KLVFF (Hetenyi et al. 2002; Tjernberg et al. 1997; Tjernberg et al. 1996b), RIIGLa (Fulop et al. 2004) and LPYFDa (Datki et al. 2003; Datki et al. 2004; Granic et al. 2010; Juhasz et al. 2009; Szegedi et al. 2005) are some of the promising starting points to develop potential drugs that can somehow reverse the negative impacts of soluble Aβ aggregates, although their underlying molecular mechanisms remain partly elusive.

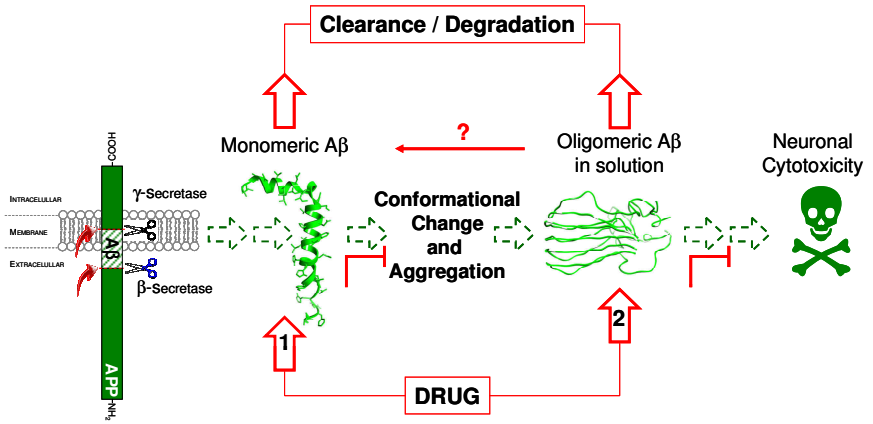
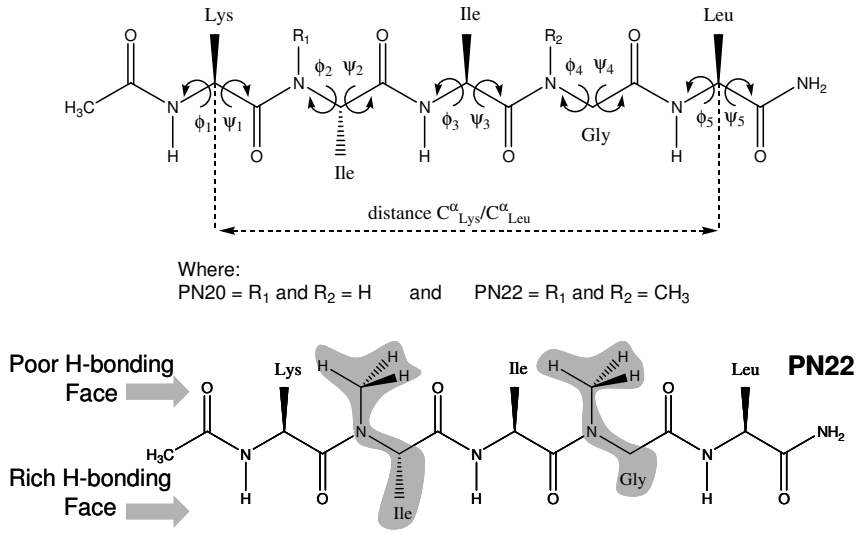


Figure 8.1: *Simplified schematic representation of the up-stream amyloid assembly process that leads to neuronal cytotoxicity. In disease states, the mechanisms of Aβ production and it clearance is unbalance causing that soluble Aβ monomers undergo aggregation to form various intermediates consisting of different forms of multimeric Aβ oligomers that, somehow, performers negative effect on brain cells. Aβ oligomers subsequently assemble to generate insoluble fibrils that accumulate in the affected tissues or organs (pathway no shown). Compounds that inhibit formation of these undesirable species may, therefore, be capable of protecting tissues or organs from their toxic effects. Two possible inhibition pathways are depicted. Pathway **1** shows an up-stream intervention of a putative drug. Thus, this drug may stop or retard the amyloigenic process by preventing the conformational change of the Aβ monomers that leads to toxic Aβ aggregates. Pathway **2** shows the intervention of a putative drug that, somehow, blocks the toxic effects of Aβ oligomers and might also reverse the amyloidic assembly process.*

In order to increase the anti-amyloidogenic properties of these peptides, a new strategy was recently explored with the introduction of N-methyl amino acids in these sequences (Cruz et al. 2004; Gordon et al. 2001; Gordon et al. 2002).

N-Methyl amino acids have been used in several systems to control, or prevent the aggregation of β-sheet and β-strand peptides (Chitnumsub et al. 1999; Clark et al. 1998; Doig 1997; Hughes et al. 2000; Nesloney and Kelly 1996; Rajarathnam et al. 1994). The goal of this modification is to block the hydrogen bond network that stabilizes the β-sheet amyloid structure and this way to inhibit the formation of toxic oligomers and/or amyloid aggregates.

In the present study, we report the *in silico* design of a new Aβ-derived synthetic penta-peptide Ac-Lys-Ile-Ile-Gly-Leu-NH_2 (PN20) and its chemically modified version; Ac-Lys-(Me)Ile-Ile-(Me)Gly-Leu-NH_2 (PN22), which contains alternated N-methyl amino acids (SCHEME 8.1).



Scheme 8.1: *Schematic representation of the chemical structure of PN20 and PN22. Backbone torsional angles and the distance $C^{\alpha}_{Lys}/C^{\alpha}_{Leu}$ are also shown. For the case of PN22 the "poor and rich H-bonding faces" are shown.*

The protective property of these peptides against the neurotoxic effects of soluble $A\beta_{42}$ oligomers was investigated in cultured mouse primary cortical neurons (PCN). Furthermore, we explored in a previously established *in vivo* model of Aβ injections directly into the hippocampus of mice whether PN22 exerts beneficial action against the Aβ-induced cognitive deficits (Granic et al. 2010). Ultimately, we intended to reveal some of the putative mechanism of action of these chemically modified peptides by employing molecular modeling techniques.

These peptides were designed based on the observations of the conformational behavior of the $A\beta_{42}$ aggregates *in silico* previously reported by Masman and coworkers (Masman et al. 2009a). The portion β2 of these aggregates, specifically the highly hydrophobic sequence Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33}-Leu^{34}, was the starting point of the present design.

8.1.1 Rational *in silico* design of PN20 and PN22

In this study we rationally designed two novel aggregation modulating peptides (PN20 and PN22). Their *in silico* design was based on the conformational behavior of $A\beta_{42}$ aggregates, which was previously reported by Masman and coworkers (Masman et al. 2009a). The so-called β2 portion of these aggregates, specifically the highly hydrophobic sequence Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33}-Leu^{34}, was the starting point of this design. Due to its hydrophobic nature some chemical modifications were

applied to the peptides in order to increase the solubility of the final peptides. Thus, the first modification was the change of the Ala residue to Lys. A positively charged residue not only increases the water solubility of the peptides but also offers the possibility of targeting important negatively charged residues that play a crucial role in the stabilization of the aggregated $A\beta_{42}$ oligomers, e.g. Glu^{22} and Asp^{23}. *A posteriori*, the N- and C-terminal were blocked by acetylation and amidation respectively. This provides some advantages: (*i*) peptide ends are uncharged (no zwitterionic), thus we have a better control on the charges that take place in a biological medium; (*ii*) increased biological membrane permeability and cell penetration; (*iii*) the stability and resistance towards digestion by aminopeptidases is enhanced; and (*iv*) peptide ends are blocked against synthetase activities. Moreover, C-terminal amidation is essential to the biological activity of many neuropeptides and hormones (Fricker 2005; Kim and Seong 2001). These two modifications were our guiding principle to the design of PN20.

In fact, a possibility to counteract the detrimental effects of oligomeric Aβ is by modulating its aggregation process. A very appealing strategy to endow peptides with aggregation modulating properties is the incorporation of non-natural amino acids to their sequence. Such is the case of N-methyl amino acids containing peptides. When the N-methyl amino acids residues are located in an alternated fashion into the peptide sequence, and the peptide has the possibility to form extended conformations, e.g. β-sheets, two different "faces" are expressed. On the one hand, one of the faces has all the normal H-bonding possibilities that a natural peptide has (the so-called "rich H-bonding face"). On the other hand, the other face is limited on H-bonding possibilities due to the presence of the N-methyl groups (SCHEME 8.1). Thus, the incorporation of N-methyl-L-isoleusine and N-methyl-glycine (or sarcosine) in position 2 and 4 respectively, by replacement of their natural analogs, led us to PN22.

8.2 MATERIAL AND METHODS

8.2.1 Molecular modeling

8.2.1.1 Stochastic and conformational search (EDMC calculations)

The conformational space of the penta-peptides PN20 and PN22 was explored using the method previously employed by Liwo et al. (Liwo et al. 1996) which includes the electrostatically driven Monte Carlo (EDMC) method (Ripoll and Scheraga 1988, 1990) implemented in the ECEPPAK package. Conformational energy was evaluated using the ECEPP/3 force field (Némethy et al. 1992). Hydration energy was evaluated using a hydration-shell model with a solvent sphere

radius of 1.4 Å and atomic hydration parameters that have been optimized using nonpeptide data (SRFOPT) (Vila et al. 1991; Williams et al. 1992). In order to explore the conformational space extensively, we carried out 20 different runs, each of them with a different random number. Therefore, we collected a total of 5000 accepted conformations. Each EDMC run was terminated after 250 energy-minimized conformations had been accepted. The parameters controlling the runs were the following: a temperature of 298.15 K was chosen for the simulations. A temperature jump of 50,000 K was used; the maximum number of allowed repetitions of the same minimum was 50. The maximum number of electrostatically predicted conformations per iteration was 400; the maximum number of random-generated conformations per iteration was 100; the fraction of random/electrostically predicted conformations was 0.30. The maximum number of steps at one increased temperature was 20; and the maximum number of rejected conformations until a temperature jump was executed was 100. Only *trans* peptide bonds ($\Theta \cong 180°$) were considered. All accepted conformations were then clustered into families using the program ANALYZE (Meadows et al. 1994; Pohorille and Pratt 1990) by applying the minimal-tree clustering algorithm for separation, using all heavy atoms, energy threshold of 30 kcal.mol^{-1}, and RMSD of 0.75 Å as separation criteria. This clustering step allows a substantial reduction of the number of conformations and the elimination of repetitions. A more detailed description of the procedure used here is given in section *4.4 Computational Methods* of reference (Masman et al. 2009b).

8.2.1.2 Molecular dynamics simulation

The most populated conformation of PN20 and PN22 obtained from the EDMC were embedded in a cubic box containing SPC waters, (Berendsen et al. 1981) leaving at least 10 Å between the solutes and the edge of the box. The total number of water molecules varied between 15630 and 25919. Cl^- ions were added to the systems by replacing water molecules in random positions, thus making the whole system neutral. Multiple simulations were performed for each system, starting from different initial random velocity distributions. Details of the equilibration procedure can be found in the Suppl. Information. For each system 10 ns production runs were obtained and analyzed. The coordinates were saved every 2 ps. Molecular dynamics (MD) simulations were performed using the GROMACS 3.3.2 package of programs (Van Der Spoel et al. 2005), with the OPLS-AA force field (Kaminski et al. 2001). The simulations were run under NPT conditions, using Berendsen's coupling algorithm (Berendsen et al. 1984) for keeping the temperature and pressure constant ($P = 1$ bar, $\Delta P = 0.5$ ps; $T = 310$ K, $\Delta T = 0.1$ ps). The LINCS algorithm (Hess et al. 1997) was used to constrain the lengths of hydrogen containing bonds; the waters were restrained using the SETTLE algorithm (Miyamoto and Kollman 1992). The time step for the simulations was 0.002 ps and

the compressibility $4.8x10^{-5}$ bar^{-1}. Van der Waals forces were treated using a 1.2 nm cutoff. Long-range electrostatic forces were treated using the particle mesh Ewald method (PME) (Darden et al. 1993). The analysis of the simulations was performed using the analysis tools provided in the Gromacs package. The root mean square deviation (RMSD) of backbone atoms, the total and potential energies were calculated. The root mean square fluctuation (RMSF) of the backbone atoms and the hydrophilic, hydrophobic and total Solvent Accessible Surface Area (SASA) were also determined. The total number of hydrogen bonds in the peptide group were quantified by counting acceptor and donor atom pairs that are not further apart than 0.35 nm. All molecular graphical presentations were created by VMD (Humphrey et al. 1996) and/or UCSF Chimera (Pettersen et al. 2004) packages. The standard deviation of every given value is shown in between brackets.

8.2.1.3 Docking studies

Two models for Aβ were used as target systems; the monomeric $A\beta_{42}$ elucidated by Crescenzi et al (monomeric model) (Crescenzi et al. 2002), PDB code 1IYT, and the pentameric aggregate $A\beta_{42}$ developed by Masman et al (pentameric model) (Masman et al. 2009a). The structures were prepared for docking study as follows: for the $A\beta_{42}$ molecules, water molecules were removed from the PDB file and hydrogen atoms were added; Gasteiger charges, atomic solvation parameters and fragmental volumes were merged to the target system. For all peptides, the structure of the most populated family (results from the *EDMC calculations*) was taken as initial conformation. Gasteiger charges were assigned and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The docking energy grid was produced with the auxiliary program AutoGrid. The grid dimensions were 61x61x61 for the monomeric model, and 90x60x60 for the pentameric model, points along the *x*-, *y*- and *z*-axes, with points separated by 0.375Å. The grids were chosen to be sufficiently large to cover significant portions of the putative binding sites. The center of the pentapeptide was positioned at the grid center. All graphic manipulations and visualizations were performed by means of the AutoDock Tools (Sanner 1999) and the Chimera (Pettersen et al. 2004) programs, and ligand docking with AUTODOCK 4 (Morris et al. 2009). The Lamarckian genetic algorithm was utilized and the energy evaluations were set at $2.5x10^{6}$. A total of 250 accepted conformations were collected. Other parameters were set to default values.

8.2.2 EXPERIMENTAL SECTION

8.2.2.1 Compounds

The peptides Ac-Lys-Ile-Ile-Gly-Leu-NH_2 (PN20), Pro-Asp-Tyr-Leu-Phe-NH_2 (scrP) and Amyloid β peptide 1−42 ($A\beta_{42}$) were purchased from EZBiolab Inc.(Carmel, USA). Anti-Aβ antibody (6E10) was obtained from Covance (Emeryville, USA). The peptide Ac-Lys-(Me)Ile-Ile-(Me)Gly-Leu-NH_2 (PN22) was purchased from AnaSpec Inc (San Jose, CA, USA). HPLC purity higher than 95% was described for all peptides used here. Other compounds used in this study were purchased from Invitrogen (Carlsbad, USA) or Sigma-Aldrich Corporation (St. Louis, USA).

8.2.2.2 Preparation of Aβ-oligomers

Oligomeric $A\beta_{42}$ was prepared as described by Granic and colleagues (Granic et al. 2010). Also, the aggregational state and the secondary structure of the oligomeric $A\beta_{42}$ preparation were examined by sodium dodecyl sulfate (SDS)-PAGE Western blotting and Circular Dichroism (CD) spectrometry. For a more detailed description of the methodology used here, see Granic et al. (2010).

8.2.3 *In vitro* testing

8.2.3.1 Primary neuronal culture

Primary cortical neurons were prepared from embryonic brains (E14) of C57Bl/6J mice. The cortices were carefully dissected, meninges were removed and the neurons separated by trituration. Cells were plated on poly-D-lysine pre-coated plates at a density of 1.2 x 10^5 cells/well (96 well plates). Neurobasal medium supplemented with 2% (v/v) B27-supplement, 0.5 mM glutamine and 1% (v/v) penicillin/streptomycin was used as a culture medium. After 48 h neurons were treated with 10 μM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and after 6 days of *in vitro* culture, the neurons were used for experiments.

8.2.3.2 Treatment of cells

Possible toxicity of the penta-peptides used here and $A\beta_{42}$ oligomers was determined by incubating neuronal cultures for 24 h with different concentrations of the peptide solutions. The neuroprotective effect of PN20 and PN22 was assessed by incubating neurons (cultured in 96 well plates) for 24 h with 25 μM oligomeric Aβ

in the presence or absence of different concentrations of PN20 or PN22 peptides. After treatments, the medium was completely exchanged, and 24 h later, the cell viability determined by an MTT-assay. All treatments were performed in triplicates and the experiments were repeated at least two times.

8.2.3.3 Determination of cell viability by MTT assay

Neuronal viability was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide] assay as described previously (Mosmann 1983). 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2 h of incubation, cells were lysed in acidic propan-2-ol solution (37% HCl/ propan-2-ol: 1/166). The absorbance of each well was measured with an automated ELISA plate reader (Bio-Rad, Munich, Germany) at 595 nm with a reference filter at 630 nm.

8.2.4 *In vivo* testing

8.2.4.1 Animals

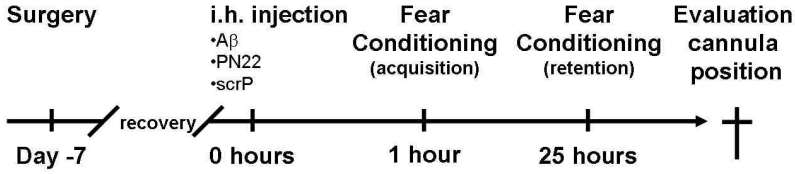
Behavioral experiments were performed with 9–12 weeks old male C57Bl/6J mice (Harlan, Horst, The Netherlands). Individually housed mice were maintained on a 12 h light/dark cycle (lights on at 7.00 a.m.) with food (Hopefarms, standard rodent pellets) and water ad libitum. A layer of sawdust served as bedding. The animals were allowed to adapt to the housing conditions for 1–2 weeks before the experiments started. The procedures concerning animal care and treatment were in accordance with the regulations of the Ethical Committee for the use of experimental animals of the University of Groningen (License number DEC4668D).

8.2.4.2 Animal surgery

Double guide cannulae type C235 (Plastics One, Roanoke, USA) were implanted in the brain using a Kopf stereotactic instrument during Hypnorm/Midazolam (10 ml/kg, i.p.) anesthesia under aseptic conditions as previously described (Nijholt et al. 2008) with anteroposterior (AP) coordinates zeroed at Bregma directed toward both dorsal hippocampi (i.h.), AP -1.5 mm, lateral 1 mm, depth 2 mm (Franklin and Paxinos 1997). Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull with dental cement (3M ESPE AG, Seefeld, Germany). Administration of 1 mg/ml finadyne (2.5 mg/kg s.c.) before the surgery served as analgesic. The animals were allowed to recover for 6–7 days before the behavioral test.

8.2.4.3 Intrahippocampal injections

Bilateral i.h. injections were performed under short isoflurane anesthesia using a Hamilton microsyringe fitted to a syringe pump unit (TSE systems, Bad Homburg, Germany) at a constant rate of 0.3 µl/min (final volume: 0.3 µl per side). Oligomerized $A\beta_{42}$ was injected in a final concentration of 30 pmol and PN22 or scrP in a final concentration of 150 pmol into the dorsal hippocampus. PBS (pH 7.5) served as vehicle. ScrP was used as a sequence control peptide. One hour after the injection the animals were subjected to a training session in a fear conditioning paradigm (SCHEME 8.2). The number of animals per group varied from 7 to 10.



***Scheme 8.2**: Schematic outline of the experimental in vivo setup. C57BL/6J mice were cannulated 7 days prior i.h. injection with oligomerized $A\beta_{42}$ and/or aggregation inhibiting peptides. One hour after the injection the animals were trained in a contextual fear conditioning paradigm. 24 h after the training session memory performance was assessed.*

8.2.4.4 Fear Conditioning

Fear conditioning was performed in a plexiglas cage (44x22x44 cm) with constant illumination (12 V, 10W halogen lamp, 100–500 lux). The training (conditioning) consisted of a single trial. Before each individual mouse entered the box, the box was cleaned with 70% ethanol. The mouse was exposed to the conditioning context for 180 s followed by a scrambled footshock (0.7 mA, 2 s, constant current) delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 s after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 h after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 s without footshock presentation. Freezing, defined as the lack of movement except for respiration and heart beat, was assessed as the behavioral parameter of the defensive reaction of mice by a time-sampling procedure every 10 s throughout memory tests. In addition, mean activity of the animal during the training and retention test was measured with the Ethovision system (Noldus, Wageningen, The Netherlands). Immediately after the behavioral test mice were injected i.h. with methylene blue solution during sodium-pentobarbital anesthesia (0.1 ml/ 10g, i.p.). Brains were removed and serially sectioned at 50 µm. Sections were stained on glass for 5 min in

0.1% nuclear fast red solution. To identify the location of the injection, sections were analyzed using light microscopy. Only data from animals in which the proper intrahippocampal site of injection was confirmed, were evaluated.

8.2.5 Statistical analysis

Behavioral data were analyzed by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test to determine statistical significance. For statistical analysis of the MTT assays, an unpaired Student's *t* test with unequal variance was used. A p-value *< 0.05 was considered to be statistically significant. A p-value **< 0.005 was considered to be highly significant. Data are presented as mean value ± standard error of the mean (SEM).

8.3 RESULTS

8.3.1 MOLECULAR MODELING

8.3.1.1 Stochastic conformational search in solution (EDMC calculations)

In order to have a better view at the molecular level, it is crucial to assess the conformational behavior of PN20 and PN22 in solution. Therefore, all peptides were selected for energy calculations to determine the relevant conformations. The results of the theoretical calculations are summarized in TABLE 8.1S in APPENDIX A. Calculations yielded a large set of conformational families for each peptide studied. The total number of conformations generated was 70236 and 79077, for PN20 and PN22 respectively, whereof 5000 conformations for each pentapeptide were accepted. In the clustering procedure, an R.M.S.D (Root Mean Square Deviation) of 0.75 Å and a ΔE of 30 kcal mol^{-1} were used. The number of families after clustering was 703 and 409, for PN20 and PN22, respectively. The total number of families accepted with a relative population higher than 0.50% was 30 and 11, for PN20 and PN22, resp. that sum up to *ca* 85% of all conformations. All low-energy conformers of pentapeptides studied here were then compared to each other. The comparison involved the spatial arrangements, relative energy and populations. The PN20 evaluation showed that the most populated family (24.20%) is also the energetically preferred one, while its second most populated family (5.52%) has a relative energy of 2.55 kcal mol^{-1} above the global minimum. The third most populated family (3.76%) shows a relative energy of 1.66 kcal mol^{-1} above the global minimum. On the other hand, the most populated family of PN22 (62.38%) is also the global minimum, while its second most populated family showed a relative energy of 0.57 kcal mol^{-1} above the global minimum and a relative population of 8.50%. The third most populated family (5.20%) has a relative energy

of 0.61 kcal mol^{-1} above the global minimum. It was observed that PN20 possesses a marked tendency to form folded conformations while PN22 shows high preference to form semi-extended or fully-extended conformations. TABLE 8.1 shows the values of ϕ and ψ torsional angles for the three most populated families of PN20 and PN22. Spatial overlapped stereo views of selected conformations, for PN20 and PN22 are shown in FIGURE 8.2.

***TABLE 8.1**: Backbone tortional angles of the three most populated families for PN20 and PN22 found at the EDMC/SRFOPT/ECCEP/3 level of theory. All angles values are given in degrees. The distance (**d**, Å) between the C^{α} atoms of residue Lys^{1} and residue Leu^{5} is also shown.*

Res.		Lys		Ile/(Me)Ile		Ile		Gly/(Me)Gly		Leu		d
		ϕ_1	ψ_1	ϕ_2	ψ_2	ϕ_3	ψ_3	ϕ_4	ψ_4	ϕ_5	ψ_5	
PN20	1	-77.93	-26.75	-77.76	-36.70	-76.80	-39.50	95.11	49.13	-76.76	-34.73	6.39
	2	-89.21	-48.10	-92.61	-50.83	-154.25	138.47	-83.89	67.60	-78.96	-41.85	9.91
	3	-74.25	-24.36	-79.41	-32.43	-76.68	-54.86	150.07	70.85	-71.13	-41.12	4.83
PN22	1	-150.42	75.40	-120.16	79.15	-140.85	73.54	-80.59	71.04	-82.85	-33.07	11.20
	2	-150.36	75.98	-117.73	83.13	-151.48	75.78	-100.57	47.66	-77.99	-34.30	11.47
	3	-150.42	76.58	-116.92	81.14	55.16	68.55	-93.90	58.60	-81.90	-32.94	9.62

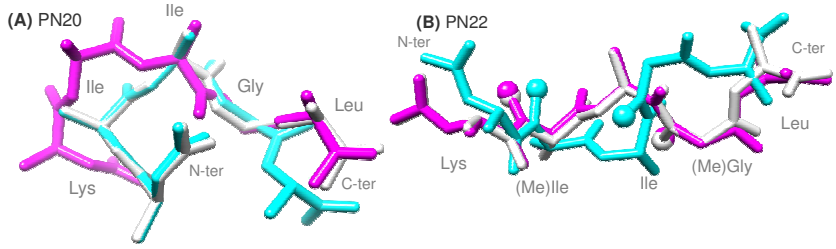


***Figure 8.2:** Stereoview of the three most populated families for PN20 and PN22 optimized at EDMC/SRFOPT/ECCEP/3 level of theory. (A) Overlapped geometries of family 1 (ΔE = 0.00 cal.mol^{-1}, white), family 2 (ΔE = 2.55 kcal.mol^{-1}, magenta) and family 3 (ΔE= 1.66 kcal mol^{-1}, cyan) for PN20. (B) Overlapped geometries of family 1 (ΔE = 0.00 kcal.mol^{-1}, white), family 2 (ΔE= 0.57 kcal mol^{-1}, magenta) and family 3 (ΔE= 0.61 kcal mol^{-1}, cyan) for PN22. All hydrogen atoms and side-chains have been deleted for more clarity. N-Methyl groups are depicted in ball-and-stick model.*

8.3.1.2 Molecular dynamics

PN20 and PN22 were examined by MD due to the need of exploring their conformational behavior in a more realistic system by means of an explicit solvent. FIGURE 8.3 shows the distance between the C^{α} atoms of residues Lys^{1} and Leu^{5} (d

C^{α}_{Lys}/ C^{α}_{Leu}) as a function of the simulation time for PN20 and PN22. This parameter is a clear and easy way of depicting the conformational preferences of these peptides. In this figure, it is observed that PN20 prefers folded conformation with a d C^{α}_{Lys}/ C^{α}_{Leu}= 4.22(0.49) Å while PN22 showed a marked preference to extended conformations (d C^{α}_{Lys}/ C^{α}_{Leu}= 12.25(1.08) Å).

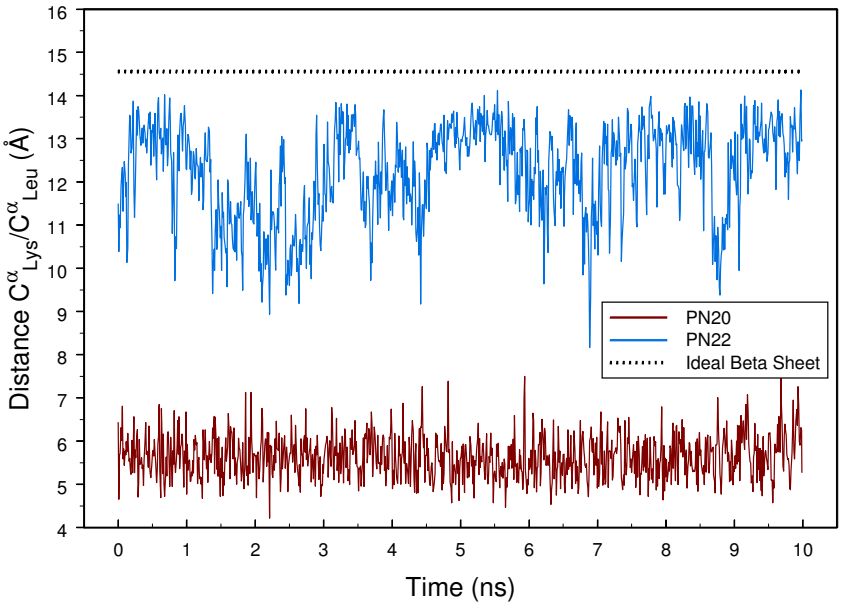


***Figure 8.3**: Distance between C^{α} atoms of residues Lys1 and Leu5 as a function of simulation time. The maximum ideal (for a fully extended conformation, where all ϕ and $\psi \equiv 180^{o}$) distance between the above mentioned atoms is also shown.*

8.3.1.3 Docking studies

The docking studies performed here have been analyzed on the basis of their conformational population distribution as a function of the binding energy. The clusterization procedure was run with a cut-off of 2.00 Å RMSD. FIGURE 8.4 shows the density population distribution for PN20 and PN22 on the two target system-models used here. *Monomeric Model*. Two potential binding sites were found by using a single blind docking run (results not shown) on the monomeric $A\beta_{42}$ molecule (PDB code 1IYT). FIGURE 8.5A depicts the location of these potential binding sites. Site I encompasses the residues 21-26 containing Glu^{22} and Asp^{23}, which were previously identified as residues for aggregation of the oligomers (Buchete and Hummer 2007; Masman et al. 2009a). Site II includes residues 6-12 located in the portion of the molecule that loses all structural organization after oligomerization, thus forming part of the so-called disordered region. The docked-conformational population for PN20 and PN22 is shown in figure 4, panel A. It

can be observed that PN20 does not show a preference to bind either Site I or Site II. PN20 obviously binds both sites unselectively and with more or less the same intensity. On the other hand, the most populated conformations of PN22 show a binding preference for Site I. Thus, the binding of PN22 on Site II is energetically weaker than the one observed on Site I. In TABLE 8.2 the two most populated families of the complexes of PN20 and PN22 with the monomeric $A\beta_{42}$ are summarized. PN22 showed lower binding energies, while site I was in general energetically preferred over site II. FIGURE 8.5B shows the interacting complex of PN22 on Site I. Amongst all the interaction observed here, it is important to highlight that the NH_3^+ group of residue Lys forms a strong salt-bridge interaction with residue Asp[23].

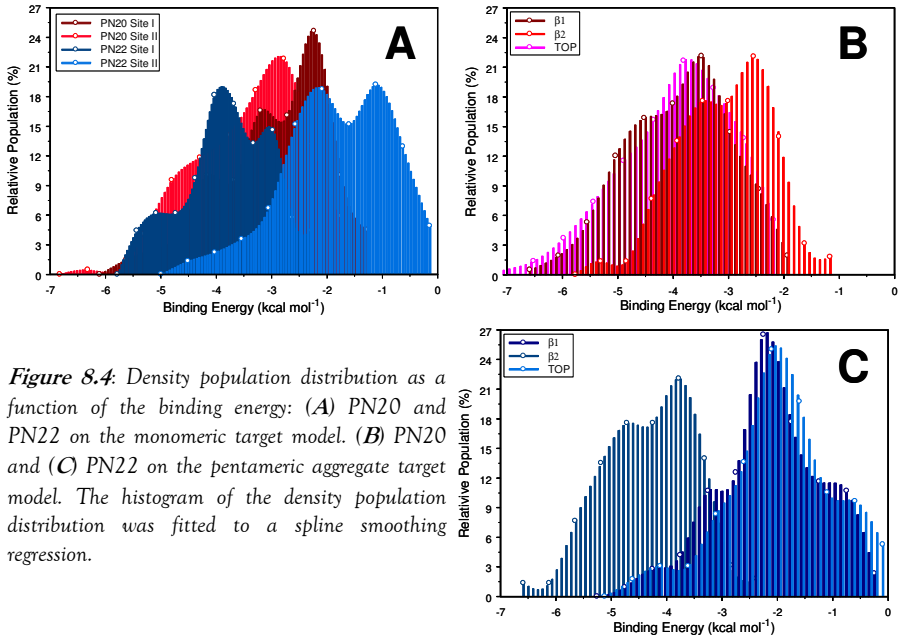


***Figure 8.4**: Density population distribution as a function of the binding energy: (**A**) PN20 and PN22 on the monomeric target model. (**B**) PN20 and (**C**) PN22 on the pentameric aggregate target model. The histogram of the density population distribution was fitted to a spline smoothing regression.*

Table 8.2: *The two most populated families of PN20 and PN22 found by docking simulations on the monomeric and pentameric Aβ_{42} peptide and the corresponding binding energy (E_B, kcal mol^{-1}) of the complexes. The binding constant (K_B, M) and the relative populations (%P) are also shown.*

			PN20			PN22		
			E_B	K_B	%P	E_B	K_B[a]	%P
Monomeric Aβ_{42}	Site I	1	-2.23	2.29x10^{-2}	24.62	-4.03	1.09x10^{-3}	18.14
		2	-3.17	4.72x10^{-3}	16.58	-3.67	2.04x10^{-3}	17.25
	Site II	1	-2.77	9.13x10^{-3}	21.82	-1.11	1.58x10^{-1}	19.20
		2	-3.28	3.88x10^{-3}	18.64	-2.08	2.98x10^{-2}	18.75
Pentameric Aβ_{42}	TOP	1	-3.81	1.56x10^{-3}	21.66	-2.10	2.88x10^{-2}	25.00
		2	-3.26	4.23x10^{-3}	18.89	-1.60	6.84x10^{-2}	19.74
	β1	1	-3.48	2.73x10^{-3}	22.12	-2.24	2.23x10^{-2}	26.51
		2	-4.00	1.17x10^{-3}	17.31	-1.74	5.23x10^{-2}	17.67
	β2	1	-2.54	1.38x10^{-2}	22.07	-4.24	7.48x10^{-4}	20.34
		2	-3.46	2.90x10^{-3}	17.57	-4.71	3.25x10^{-4}	19.49

[a] K_B is calculated in with the equation K_B=exp((ΔG*1000.)/(Rcal*TK), where ΔG is the docking energy, Rcal is 1.98719 and TK is 298.15

Pentameric Model. The target model used here was previously reported by Masman and coworkers (Masman et al. 2009a). This Aβ_{42} aggregates model contains two β-sheet moieties (β1, residues 18-26 and β2, residues 31-42), which are proposed as putative binding sites. Moreover, a third possible site for interactions with ligand molecules is postulated, which involves both above mentioned sites (β1 and β2) at the edge of the aggregate. This site, here called the TOP site, is orientated into the oligomeric axis, in the same direction where the oligomers grows by aggregation. TABLE 8.2 shows the two most populated families of the complexes of PN20 and PN22 with the pentameric Aβ_{42}. It is observed in FIGURE 8.4B that PN20, in contrast with our expectation, has no preference to bind any of the sites targeted here. PN20 unselectively binds to β1, β2 and TOP sites. On the other hand, PN22 displayed a preference to bind the β2 site compared to the other two proposed sites.

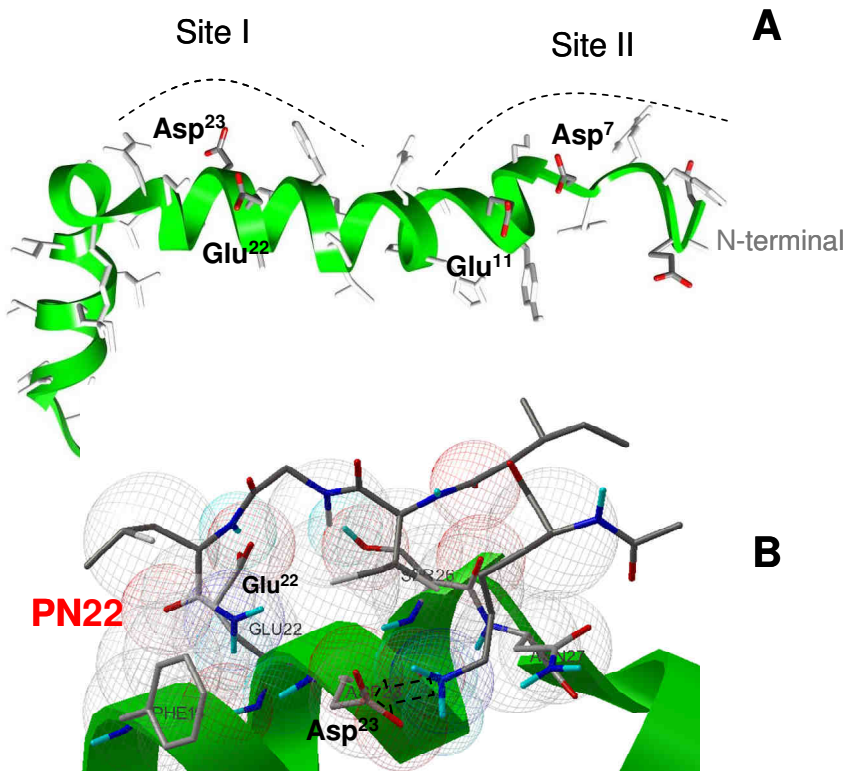


***Figure 8.5**: (**A**) Location of site I and site II on the monomeric Aβ42 model. (**B**) Stereoview of the populated complex PN22/$A\beta_{42}$ monomeric on site I. All ligand-target contact are depicted as wireframe spheres. Salt-bridge Lys-NH_3^+/Asp^{23} is marked with a block-arrow.*

FIGURE 8.4C shows that the most populated conformations are energetically preferred when PN22 binds to site β2, while this peptide binds nonspecifically to β1 and TOP sites. FIGURE 8.6A illustrates an ideal H-bonding arrangement for TOP interaction of PN22 with an Aβ aggregate. It is depicted that PN22 may expose a "rich H-bonding face" to interact with the target molecule while exposing a "poor H-bonding face" that may stop the aggregation process. FIGURE 8.6B shows the atomic details of the interactions of the most populated complex (family 1 TOP, in TABLE 8.2). This interacting complex is the most similar arrangement to the ideal case. Even though the quantity of H-bonds was less abundant than in the ideal case, the orientation of the "poor and rich" H-bonding faces appear similar. FIGURE 8.7 reveals the atomic details of the interactions of the second most populated complex (family 2 β2, in TABLE 8.2) found between PN22 and the pentameric $A\beta_{42}$. All the ligand-target contacts are depicted as wireframe spheres.

PN22 is located in a transversal direction to the Aβ chains, and inside an important channel-like hydrophobic pocket formed between the residues Ile[31] and Met[35] of the aggregate. The side-chains of PN22 are localized parallel to the Aβ backbone. Thus, the side-chains act as hydrophobic anchors. Obviously, due to the highly hydrophobic nature of both, target and drug molecules, the predominant interaction type is the hydrophobic contact. Also, it is interesting to mention that the N-methyl groups face the Aβ aggregate, this way increasing the hydrophobic contact between drug and receptor. Accordingly, the "rich H-bonding face" is exposed to the surrounding space, where the solvent (water) should be.

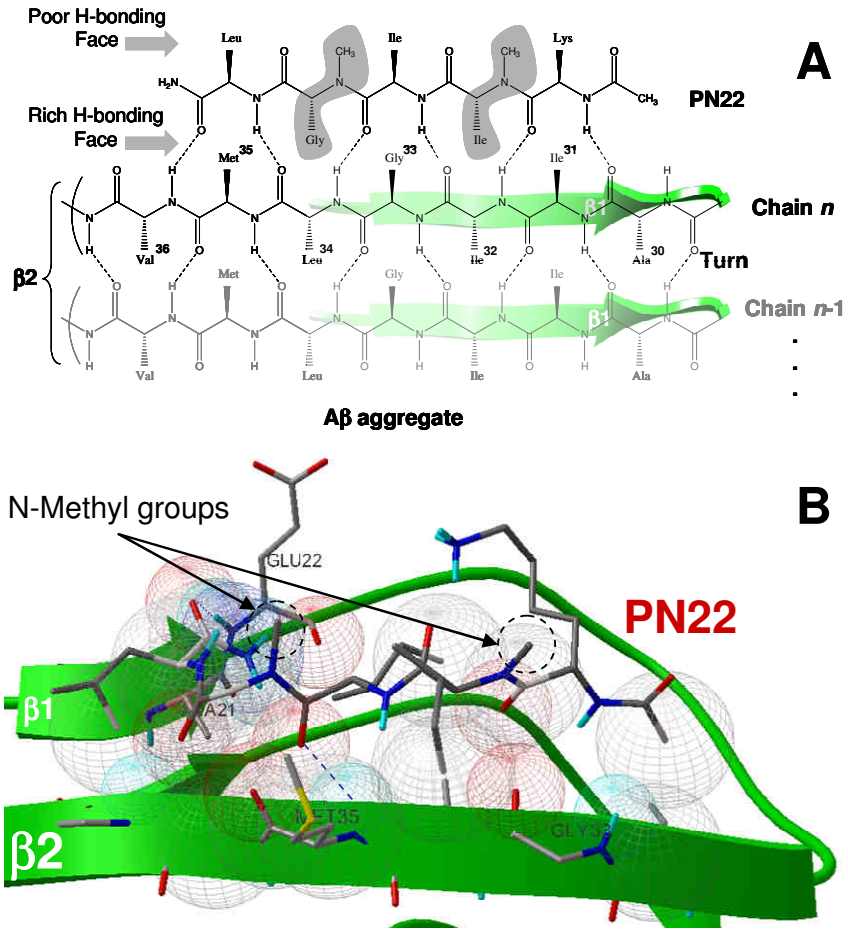


Figure 8.6: (*A*) *An ideal TOP interaction of PN22 with an* $A\beta_{42}$ *aggregate. The N-methyl amino acid residues are shaded in grey. Hydrogen bonds are represented as slashed lines (--). (B) A steroview of most populated complex PN22/Aβ42 pentameric model on site TOP. All ligand-target contact are depicted as wireframe spheres.*

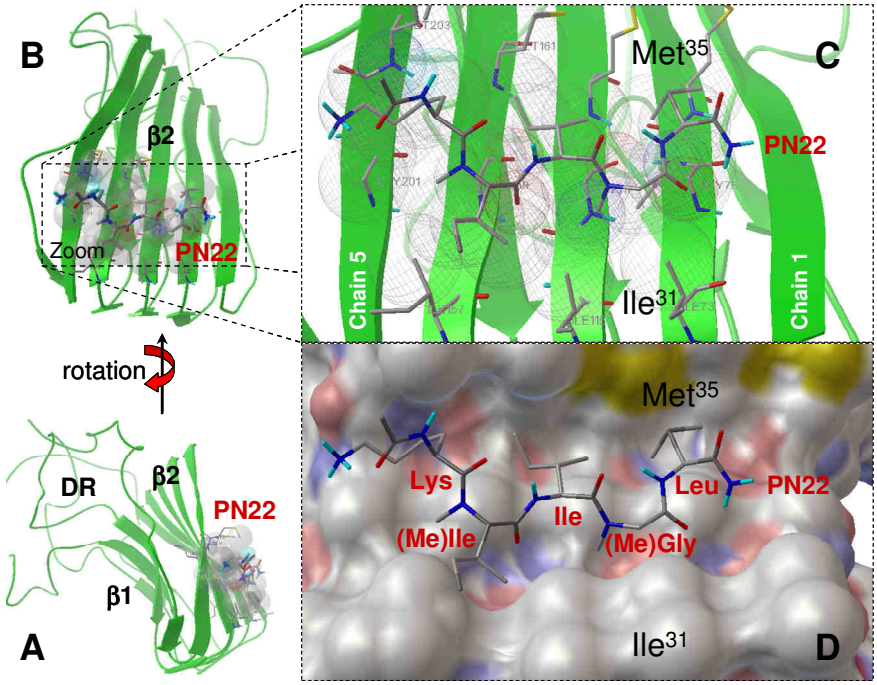


***Figure 8.7**: A stereoview of the second most populated complex PN22/Aβ_{42} pentameric on site β2. Different views are shown: lateral (**A**), frontal (**B**), zoomed in (**C** and **D**). Panel **C** shows the ligand-target contact which are depicted as wireframe spheres. Panel **D** shows a steroview of PN22 in the channel-like hydrophobic pocket formed, mainly by residues Ile^{31} and Met^{35}.*

8.3.2 EXPERIMENTAL TESTING

8.3.2.1 PN22 is neuroprotective against oligomeric Aβ_{42} *in vitro*

Part of this study was to provide proof of principle that the newly designed aggregation modulator peptides have the potential to protect nerve cells against Aβ-induced toxicity. Thus, we tested firstly whether these novel peptides exhibited any toxicity to neuronal cultures by themselves, and secondly whether PN20 and PN22 were capable to neutralize the toxic effect of oligomeric Aβ_{42}. To this purpose primary cortical neurons in culture were exposed to different concentrations of PN20 or PN22 alone, to Aβ_{42} alone (25 μM) or to pentapeptides and Aβ_{42} together for 24 h. Neither PN20 nor PN22 alone was toxic to neurons at any tested concentration (FIGURE 8.8). When exposed to 25 μM oligomeric Aβ_{42} only 30-40 % of the cultured neurons survived a 24 h incubation. Furthermore, both PN20

and PN22 were able to protect the neurons from $A\beta_{42}$-induced toxicity in a dose dependent manner (FIGURE 8.8). The methylated PN22 (FIGURE 8.8B) in that respect proved to be more effective reaching significance at 8 µM; P=0.02 when compared to the non-methylated PN20 (FIGURE 8.8A) (reaching significance at 32 µM; P=0.01). With the highest concentration (250 µM) of PN20 up to 62.3± 12.9% of the neurons survived the Aβ treatment. 250 µM of PN22 was even more protective against the $A\beta_{42}$ challenge with up to 86.3± 3.7% cell survival.

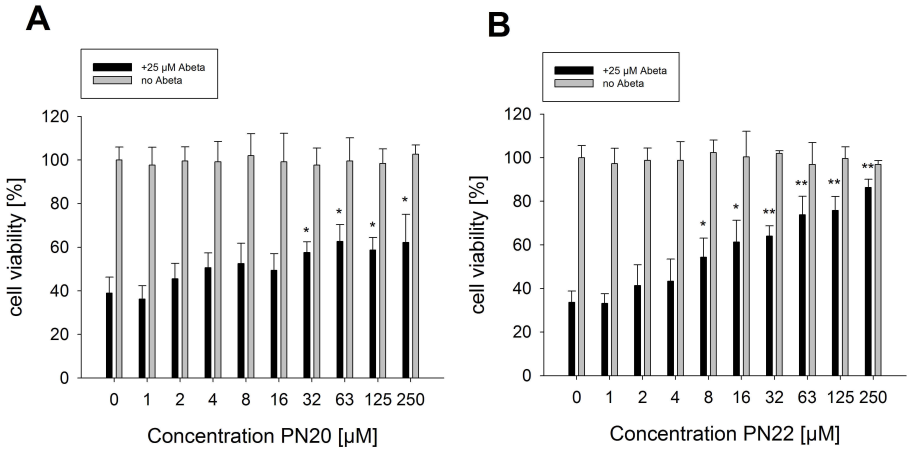


***Figure 8.8**: Cell viability of primary cortical neurons determined by an MTT-assay. Neuronal survival was determined after incubating increasing concentrations of the pentapeptides (**A**) PN20 and (**B**) PN22 with or without 25 µM $A\beta_{42}$ for 24 h. Bars indicate the mean cell viability in % relative to untreated controls ± SEM. (* = significant at $p < 0.05$; ** = highly significant at $p<0.005$).*

8.3.2.2 Cognitive deficits induced by oligomeric $A\beta_{42}$ can be neutralized by PN22

Based on the *in vitro* data we selected the most effective peptide PN22 and tested whether PN22 is able to reverse the Aβ-induced memory deficits. We further included a scrambled peptide (scrP) as a control for sequence specificity of the peptides. To that purpose we used an animal model in which we injected mice with oligomeric $A\beta_{42}$ into the hippocampus to induce memory deficits (Granic et al., 2010).

One hour prior to the training session in a contextual fear conditioning paradigm C57BL/6J mice received a single injection of oligomeric $A\beta_{42}$ (30 pmol) or vehicle with PN22 (150 pmol) or scrP (150 pmol). In general the injections did not affect locomotion or the shock reaction during training (data not shown). The vehicle injected animals displayed an average relative freezing behavior, as a measure of

memory score of 57.1± 2.2%, which did not differ from untreated control animals (58.1± 3.6 %). The injection of 30 pmol oligomerized $A\beta_{42}$ led to a significant decrease in freezing behavior compared to the vehicle group (33.7± 6.5% vs 57.1± 2.2; p=0.002) (FIGURE 8.9) indicative of impaired memory storage.

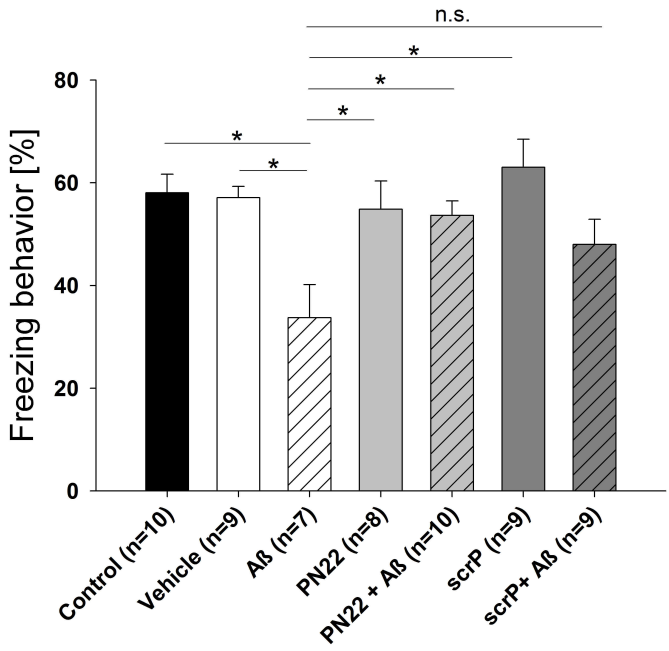


***Figure 8.9**: Effect of $A\beta_{42}$ and PN22 on contextual fear conditioning. Co-injection of $A\beta_{42}$ with PN22 prevented the $A\beta$-induced memory impairment significantly, whereas the non-specific control peptide scrP failed to revert memory deficits. Bars indicate the mean relative freezing score in % ± SEM. Differences were determined by ANOVA (* = significant at $p < 0.05$).*

The PN22 and the scrP injected mice showed an average freezing of 54.9± 5.0% and 63.0± 5.5% respectively, which did not significantly differ from the untreated control (61.1± 2.4%) or vehicle injected group (58.9± 2.9%).

However, PN22 co-injected with $A\beta_{42}$ was able to abolish the $A\beta_{42}$ oligomer-induced memory impairment (53.7± 2.8% vs 33.7± 6.5%; p=0.01). Co-injection of Aβ with scrP resulted in an average freezing score of 48.0± 4.9%, which did not significantly reverse the Aβ-induced memory deficits. These results provide evidence that PN22 is capable to neutralize the negative effects induced by $A\beta_{42}$ oligomers on memory performance in an *in vivo* test paradigm.

8.4 DISCUSSION

Crucial for the aggregation process of the Aβ molecule are the hydrophobic residues that encompass the β2 region (amino acids 30-42). Thus, it was also observed for $A\beta_{42}$ monomers in solution that the sequence $I^{31}IGLMVGGVVIA^{42}$ (namely, the β2 portion) may be responsible for the higher propensity of this peptide to form amyloid aggregates (Sgourakis et al. 2007). Therefore, small peptides like PN20 and PN22, which are partially homologous to this hydrophobic region, bind with a relatively high affinity to Aβ by similar intermolecular interactions, leading to a competitive replacement of Aβ molecules. Interestingly our docking results showed that PN22 binds preferably to the β2 portion of the aggregate (TABLE 8.2 and FIGURE 8.4C), which has a good correlation with the design of this pentapeptide. However, in contrast with our expectations, PN20 did not show such a binding profile. This may be explained by the fact that PN20 displayed a complete different conformational behavior in solution than PN22 as shown by EDMC and MD simulations. PN22 had a strong preference for extended conformations, while PN20 showed a strong affinity to folded conformations. This was not only observed in solution but also in our docking results. Thus, an extended or semi-extended conformation was observed for the majority of the most populated complexes that PN22 formed, whereas the opposite was the case for PN20. It appears to be that an extended or semi-extended conformation is the possible "biologically relevant conformation" or "pharmacophoric patron" for these peptides.

On the other hand, all the docked preferred conformations showed a tendency to extended or semi-extended ligand's orientation. Moreover, the docking studies could predict that a modification of PN20 by alternated N-methylation, thereby moderately increasing its hydrophobicity, improves the interaction to the β2 portion of the $A\beta_{42}$.aggregates. Thus, PN22 shows a selective binging to the portion β2. Neither PN20 nor PN22 showed a preference to bind the monomeric or the pentameric $A\beta_{42}$ model, which suggests us that the peptides may bind to both forms of $A\beta_{42}$.

We hypothesize that Site I-bound-PN22, especially on Asp^{23}, causes impediment or slowing down of the conformational change that precedes the aggregation. Consequently, PN22 binding increases the possibilities of degradation or clearance of Aβ monomers (see FIGURE 8.1), since, Asp^{23} plays a crucial role in the stabilization of the Aβ aggregates. In general it can be observed that PN22 binds stronger and selectively to Site I, to the monomeric $A\beta_{42}$ than PN20.

The present results indicated that pentapeptides PN20 and PN22 can significantly preserve cultured neurons from Aβ-induced cell death in a dose dependent manner. Moreover, the methylated pentapeptide PN22 protected the cells more effectively

than the non-methylated PN20. Similar results on PC12 cells by using different single N-methyl amino acid containing peptides were previously reported (Cruz et al. 2004; Hughes et al. 2000). Peptides endowed with N-methyl amino acids have some clear advantages with respect to their potential as a therapeutic agent. These peptides remain remarkably stable to changes in solvent conditions and resist denaturation by heating, changes in pH (from 2.5 to 10.5), and addition of denaturants. Furthermore, this kind of peptides despite their hydrophobic composition is highly water soluble. A water soluble but hydrophobic nature suggest that these peptides might be able to pass spontaneously through biological cell membranes (Gordon et al. 2002).

An important aim of our study was to establish if a compound like PN22 is able to prevent Aβ–induced learning and memory deficits in a mammalian animal model. We showed that a 5-fold molar excess of PN22 to Aβ could overcome the detrimental effects of Aβ oligomers on memory when injected into the hippocampal region. Thus, we provide evidence that N-methyl containing peptides such as PN22 bear therapeutic potential against Aβ-induced memory impairment.

The mechanism how these pentapeptides exert their protective effects on cell death and behavior is not yet fully understood. However, these peptides directly bind to Aβ and thereby may prevent possible interactions between Aβ and neuronal membrane proteins and in this way neutralize the toxic effect of Aβ oligomers. We hypothesize two options of interaction notably of PN22 with Aβ that are depicted in figure 1. Pathway 1 proposes that PN22 binds to the monomeric Aβ thus preventing and/or retarding the formation of toxic oligomers, by interfering with the conformational change that precedes the oligomer formation. Pathway 2 suggests that PN22 binds to the already formed Aβ oligomers thereby preventing and/or modulating, somehow, its neurotoxic properties. By either of these actions, or both, the neurotoxicity of Aβ is decreased or reverted and the Aβ clearance/degradation processes be reactivated.

We may conclude that this novel *in silico* designed N-methyl amino acid containing peptide interacts with $A\beta_{42}$ and that different regions of Aβ may be selectively targeted by this peptide. Our findings provide evidence on how and where PN22 interacts with $A\beta_{42}$ mono- and oligomers, that PN22 can neutralize the neurotoxic effects of soluble $A\beta_{42}$ oligomers *in vitro* and *in vivo*. In the latter condition we obtained proof of principle that this pentapeptide can effectively prevent the Aβ oligomer-induced deficits in memory performance. More structural evidence is required to consolidate the proposed mechanism and to further improve our peptide designs. Nevertheless, it is clear that these peptides can serve as promising molecular designs that hold promise as therapeutic molecules for treatment of AD.

CHAPTER 9

Summary and General Discussion

ABSTRACT

AD is a heterogeneous multifactorial disease, in which many different molecular mechanisms contribute to its pathogenesis. Even 100 years since the first description of the disease by Alois Alzheimer, the cause for the disease is still not exactly known.

So far, ageing, oxidative stress, disrupted ion homeostasis, inflammation, and genetics have been identified as major contributors to the pathogenesis, which might be up- or downstream of the well-known hallmarks of the disease: the Aβ plaques and neurofibrillary tangles. As such all of those factors provide possible targets for intervention in the process of the disease initiation and progression.

Here I will shortly elaborate on different therapeutic strategies, which are based on our current understanding of the pathogenesis of AD. The approaches used here, engage several molecular mechanisms underlying the disease process, such as neuroinflammation, neuronal death, Aβ aggregation and associated toxicity and memory impairment.

9.1.1 Neuroinflammatory TNF signalling as therapeutic target in Alzheimer's disease

Next to plaques and tangles, neuroinflammation is another pathological feature of AD and is characterized by activated glia cells and notably microglia. These cells can be regarded as the "immune cells" of the brain which secrete a large variety of factors, that can be trophic or toxic, depending on the target cell and on the activated receptor systems involved. One of the most potent factors released by microglia is the pro-inflammatory cytokine TNF, which is increased in many neurodegenerative disorders including AD. It is still under debate whether inflammation and associated elevated cytokine levels have beneficial or detrimental effects for the diseased brain.
Several studies showed that TNF overexpression in mice leads to detrimental effects on synaptic plasticity resulting in impaired learning and memory (Aloe et al., 1999; Fiore et al., 2000). Moreover, TNF receptor signalling is involved in APP processing and Aβ production. Several groups have identified an NF-κB binding –site in the β-secretase (BACE1) promoter region (Christensen et al., 2004; Li et al., 2006b). This could implicate that sustained NF-κB activation might lead to elevated β-secretase levels and increased Aβ production. Indeed, He and colleagues demonstrated that TNF treatment increases BACE1 activity *in vivo* which is mediated via TNFR1 (He et al., 2007).

9.1.2 TNF as drug target in AD

On the other hand TNF can protect adult neurons after traumatic brain injury or ischemic insults (Fontaine et al., 2002; Marchetti et al., 2004; Sullivan et al., 1999). Transgenic mice deficient for TNFR2 showed much larger damage in a model for retinal ischemia. In contrast transgenic animals lacking TNFR1 are well protected against ischemic damage (Fontaine et al., 2002).
These and other studies suggest that modulation of the distinct TNFR signalling pathways holds therapeutic potential for the treatment of AD. So far anti-TNF agents have been used in clinical trials to treat heart failure and rheumatoid arthritis with mixed outcome. Results from smaller pilot studies using anti-TNF approaches, were encouraging, however larger scaled studies failed to prove clinical benefits (Dhillon et al., 2007; Mann et al., 2004). One of these anti-TNF agents is known under the name etanercept, which is a recombinant, soluble human TNF receptor that binds and neutralizes soluble TNF. Etanercept has received much attention in the scientific community, when in 2006 Tobinick and coworkers reported on beneficial effects of etanercept if administered perispinally in moderate to severe AD patients (Tobinick, 2007; Tobinick et al., 2006). To evaluate whether the use of etanercept in AD patients has really beneficial effects, larger clinical trials are needed.

Another potential strategy for many neurodegenerative disorders involves the selective activation of the neuroprotective TNFR2 signalling. In an *in vitro* model of glutamate-induced cell death of primary cortical neurons, TNF was shown to be protective via TNFR2, PKB/Akt and NF-κB signalling (Dolga et al., 2008; Fontaine et al., 2002; Marchetti et al., 2004). Although the involvement of TNFR2, PKB/Akt and NF-κB was already suggested, only limited data are available on putative neuroprotective downstream targets of NF-κB.

A potential mechanism underlying the TNFR2 mediated protection against glutamate toxicity was dissected in **chapter 3** of this thesis. Here I show that the protective effect of TNF against a glutamate challenge was accompanied by a selective, NF-κB dependent increase in KCa2.2 channels. The expression of the KCa2.2 channels could be blocked by the specific inhibitor of NF-κB (BAY11-7082). It is very likely that NF-κB interacts with one of two NF-κB binding sites, which were recently identified as regulatory parts of the murine KCa2.2 promoter (Kye et al., 2007).
Furthermore, the increased expression of KCa2.2 channels after TNF treatment was mediated by TNFR2, which corresponds well with previous findings that TNFR2 mediates TNF-induced neuroprotection (Dolga et al., 2008; Fontaine et al., 2002; Marchetti et al., 2004).
In the context of neuroprotection KCa2.2 channels can mediate a decrease in neuronal excitability which is likely part of the protective mechanism. Indeed, the neuroprotective potential of KCa2.2 was observed in an earlier study showing that over-expression of KCa2.2 channels in cultured hippocampal neurons increases resistance against a kainic acid induced excitotoxicity (Lee et al., 2003).

In general, the overstimulation of neurons by excitatory neurotransmitters can trigger long lasting activation of ionotropic receptors (e.g. NMDA, AMPA), which can lead to excessive Ca^{2+} influx into the cell. Prolonged increase of intracellular Ca^{2+} can eventually result in neuronal cell death and functional impairment of affected brain areas.
As a physiological response to changes of intracellular Ca^{2+} concentrations KCa2.2 channels can be activated to induce a re-polarizing effect on the membrane. This re-polarizing action of KCa2.2 can oppose the depolarizing effect of AMPA receptor activity, favouring Mg^{2+} re-blocking of NMDA receptors and thus reducing the Ca^{2+} transient (Faber et al., 2005; Lin et al., 2008; Ngo-Anh et al., 2005). In this way KCa2.2 channels may decrease glutamate-induced excitotoxicity which may lead to reduced susceptibility to glutamate-induced over-stimulation via their coupling to NMDA receptors.

As described in the previous sections it becomes clear that neuroinflammation, neurodegeneration and neuroprotective signalling intersect at the molecular level of the nuclear transcription factor NF-κB. NF-κB is involved in a large variety of cellular processes and plays a central role in the initiation and amplification of inflammation by responding to proinflammatory stimuli such as TNF or

interleukin-1 (IL-1) (Hayden et al., 2006; Karin and Greten, 2005; Tak and Firestein, 2001). The aberrant regulation of NF-κB leads to the development of many pathological states especially those involving acute inflammation such as AD and diabetes mellitus (DM) (Kaltschmidt et al., 1997; Katarina et al., 2007).

9.2 Inflammation and NF-κB as link between AD and Diabetes

Inflammatory processes are a hallmark of many chronic diseases including AD and DM. Statistical evidence published in the Rotterdam study and but also several other studies linked DM with an increased risk to develop AD (Biessels et al., 2006a; Biessels et al., 2006b). Such evidence encouraged researchers worldwide to search for the causal mechanisms underlying this relationship.

In **Chapter 6** I provide an overview on the current literature linking AD and DM via inflammatory processes and NF-κB. Because the NF-κB pathway plays a major role in the etiology in both diseases, it seems reasonable to assume that mechanisms causing disturbances in this pathway are at the core of the relationship. From the present literature it seems obvious that inflammatory processes are playing essential roles in the etiology of T2DM and AD. Aberrant regulation of the inflammatory pathway involving NF-κB, which includes TNF dependent and TNF independent mechanisms, directly underlies insulin resistance in peripheral tissue as well as in astrocytes in the brain. Since insulin resistance in the periphery can lead to glucose intolerance, neuronal inflammatory processes triggering (or triggered by) the NF-κB pathway may be propagated even further via stimulation of the AGE/RAGE signalling pathway. Therefore, it might be expected that alleviating symptoms of T2DM may be an effective way to treat AD. Indeed, recent clinical trials with the insulin sensitizer rosiglitazone -i.e., an agonist for the peroxisome proliferator-activated receptor gamma (PPARγ), (whose biological actions are to regulate glucose and lipid metabolism and suppress inflammatory gene expression) have shown a significant improvement in memory and cognition in AD patients (Landreth et al., 2008).

9.3 Statins and Alzheimer's Disease

Despite contradictory results reported in the recent literature, increasing evidence points to elevated cholesterol levels to enhance the risk to develop AD (Martins et al., 2009; Kivipelto et al., 2002; Panza et al., 2006; Pappolla et al., 2003). In fact cholesterol metabolism and catabolism are affected in many neurodegenerative disorders including AD. Since APP processing and subsequent Aβ production are dependent on membrane cholesterol content and on levels of isoprenoid intermediates in the cholesterol biosynthesis pathway, changes in cholesterol might have profound consequences on Aβ generation and the course of the disease. These pieces of evidence support the idea that inhibitors of cholesterol synthesis, like

statins could have a therapeutic role in AD. The following section discusses the potential role of statins as potential therapeutic for neurodegenerative disorders.

9.3.1 Statins modulate neuroinflammatory processes

Statins are widely prescribed as cholesterol lowering drugs and the use of statins is associated with a decreased risk for AD, although the mechanisms are poorly understood. However, recent studies have shown that statins have pleiotropic actions, which are not solely dependent on cholesterol reduction (Cole and Vassar, 2006; Liao and Laufs, 2005). Dolga and co-workers could show in an *in vitro* study that lovastatin induces neuroprotection against glutamate induced excitotoxicity through TNFR2 mediated signalling (Dolga et al., 2008). However, from that study it remained unclear whether beneficial effects of lovastatin *in vitro* could be translated into an *in vivo* situation.

Chapter 2 of this thesis provides evidence that mice pre-treated with lovastatin, are protected from NMDA-induced excitotoxic lesions of the nucleus basalis magnocellularis. Interestingly, lovastatin treatment fully prevented behavioral deficits associated with the cholinergic fiber loss. In contrast to acetylcholinesterase inhibitors, lovastatin is able to preserve cholinergic neurons from excitotoxic damage instead of "just boosting" the amount of available neurotransmitter and thereby improving cognitive performance.

These results also show that the neuroprotective effect of lovastatin is dependent on the activation PKB/Akt signalling, since neuroprotection could be abolished by a selective inhibitor of phosphoinositide 3-kinases (PI3K).

It is unlikely that behavioral alteration of lovastatin-treated animals can be attributed to direct drug effect since statins are rapidly cleared from the brain within 6 hours after drug administration (Johnson-Anuna et al., 2005). All behavioral tests were performed within 7 days after the last lovastatin administration. Statins such as lovastatin are able to cross the blood brain barrier and reach the cerebral cortex within one hour after drug administration but are significantly reduced 6 hours later after a single drug application. It is not clear whether the effects of statins as found in the present study can be directly associated with cholesterol dependent processes in brain tissue.

Several studies report on brain cholesterol levels after statin treatment with conflicting results. One study, for example shows that long-term treatment of simvastatin for 3 months did not change the total cholesterol levels in the brain, although the plasma total cholesterol levels were strongly reduced (Li et al., 2006a). Other studies report on reduced brain cholesterol levels in young C57Bl/6J mice treated for 3 weeks with simvastatin or pravastatin, but not with lovastatin (Johnson-Anuna et al., 2005).

Beside the cholesterol lowering effects, statins might exert their neuroprotective actions via alternative mechanisms. Recently, Ma and co-workers reported that statins can activate the non-amyloidogenic cleavage of the APP by increasing α-secretase activity (Kojro et al., 2001; Ma et al., 2009). This leads to increased

secretion of sAPPα which also mediates protection against excitotoxicity (Kojro et al., 2001). Interestingly, LY 294002 not only abolishes the statin mediated protective effect against NMDA, but also attenuates its effect on sAPPα secretion (Ma et al., 2009).
The pleiotropic actions of statins (and lovastatin in particular) might be attributed to the inhibition of the isoprenoid pathway, thereby inhibiting the activities of the Rho family of small GTPases-Rho A (Buxbaum et al., 2001; Ostrowski et al., 2007; Pedrini et al., 2005; Puglielli et al., 2001). Isoprenylated Rho proteins exert many of their effects via Rho-associated protein kinases (ROCKs). ROCK can phosphorylate substrates like PTEN and stimulates its phosphatase activity, which in turn antagonizes PI3K activity, resulting in reduced Akt phosphorylation (Li et al., 2005) and NF-κB activation.

In the CNS, active NF-κB has a pro-survival effect on neurons and can counteract apoptotic signals. Inflammatory molecules such as TNF are capable to trigger the NF-κB signalling pathway and thereby deploy its beneficial effects. In this way the modulation of neuroinflammatory processes via TNF has interesting therapeutic potential for treatment of neurodegenerative disorders.

Whereas the previous sections dealt with drug targets related to inflammatory processes and the impact of statins, the next part of the discussion will focus on a neuroprotective approach at the very end of downstream pathological events; namely the neuroprotection via the inhibition of calpains.

9.4 Calpain inhibition mediates neuroprotection against Aβ and excitotoxicity induced death

A number of authors have suggested that the pathological effect of Aβ oligomers is mediated via the over-activation of the NMDA receptor (DeFelice and Goswami, 2007; Kelly and Ferreira, 2006; Shankar et al., 2007), and that this excitotoxicity should be considered as a common principle for some major neurological diseases, including AD (Harkany et al., 2000; Lipton and Rosenberg, 1994; Mattson, 2004).
Excitotoxic stimulation via the NMDA receptor leads to excessive entry of Ca^{2+} into the cell and eventually leads to the activation of the calcium-activated cystein protease calpain, which has been implicated in pathways ending in programmed neuronal cell death. Caba and colleagues could demonstrate that the inhibition of calpain provides protection against NMDA-exposure in hippocampal slice cultures (Caba et al., 2002). Another study revealed that calpain inhibition reduces neurodegeneration in the rat retina after NMDA-injection (Chiu et al., 2005). Calpain has therefore been discussed as a promising target for treatment of neurodegenerative disorders (Huang and Wang, 2001; Saez et al., 2006; Zatz and Starling, 2005). In **chapters 4 and 5** I tested this hypothesis and provide evidence that calpain inhibition is, indeed, neuroprotective against different toxic stimuli *in*

vivo. Injection of NMDA or oligomeric Aβ to the nucleus basalis of rats and mice respectively caused a strong deterioration of cortical cholinergic projections leading to deficits in learning behavior. The cholinergic denervation in the parietal cortex and the associated behavioral decline were prevented by the application of the calpain inhibitor A-705253. Moreover, the extent of microglia activation around the injection site in the NBM was significantly reduced in animals pretreated with A-705253.

Interestingly, the *in vitro* studies in **chapter 5** using Aβ indicate that calpain inhibition is not only effective when the compound is applied prior to the insult. Efficacy of A-705253 against Aβ-induced cell damage was demonstrated up to 1 hour after administration of Aβ. This indicates that the Aβ-induced cell death cascade – once initiated – may not instantly kill the neuron, but requires some time until degeneration is irreversible. If this holds true also for other acute forms of neurodegeneration, this could offer a promising avenue for the treatment of acute neurodegenerative disorders.

The calpain inhibitor A-705253 was introduced by Lubish and co-workers (Lubisch et al., 2003) and does not affect caspases or the proteasome (Ki calpain=27 nM; Ki proteasome>10000 nM; Ki caspases>10000 nM). Therefore, it is likely that the protective effects against NMDA and Aβ-oligomer toxicity are mediated by inhibition of calpain, and not by reducing proteasome or caspase activity. The proteasome complex (Pan et al., 2008; Rubinsztein, 2006) as well as caspases (Cribbs et al., 2004) potentially contribute to neurodegeneration, and a discrimination from these targets mandatory. Battaglia and colleagues by using another, less specific calpain inhibitor, E64 (Battaglia et al., 2003), showed that long-term (5 months) intraperitoneal application of E64 in APP(K670N/M671L)/PS1(M146L) mice prevented synaptic impairment as measured by LTP. Chronic calpain inhibition also attenuated the development of deficits in spatial behavior of these mice. Recently Kelly and colleagues revealed the involvement of calpain in Aβ-mediated dysfunction of the synaptic vesicle machinery (Kelly and Ferreira, 2006; Kelly et al., 2005). Thus, it is likely that calpain inhibition serves to protect from acute and chronic neuronal decline, and from a variety of pathological features observed in AD.

In summary calpain inhibition significantly decreases acute NMDA and/or Aβ oligomer-induced degeneration of NBM and its associated cholinergic fiber projections, and attenuates behavioral deficits associated with such lesions. Furthermore, the *in vitro* data suggest that inhibition of calpain is still effective when performed shortly after the insult. These findings indicate that inhibiting calpain could be a promising strategy for the therapeutic intervention of excitotoxic and Aβ related neuropathology including AD.

Although statins and calpain inhibitors have different modes of action their neuroprotective mechanisms intersect on several molecular levels. Statins, in particular lovastatin, seem to intervene quite early/upstream in the sequence of pathogenic events, due to their pleiotropic actions (Cole and Vassar, 2006; Liao and

Laufs, 2005). Dolga et al showed that lovastatin stimulates selectively the expression of TNFR2 in primary cortical neurons which provides protection against excitotoxic insults (Dolga et al., 2008). This study implicates that the use of lovastatin might be beneficial under neurodegenerative conditions such as in AD by stimulating neuroprotective TNFR2 signalling. During neuronal insult and subsequent disruption of Ca^{2+} homeostasis, conversion of inactive p35 to active p25 is mediated via proteolytic cleavage by the calcium-regulated calpains. Recently, CDK5 has received considerable attention as a regulator of neuronal death (Cruz and Tsai, 2004). In particular, studies using apoptotic and excitotoxic cell death models demonstrate that p25/CDK5 complexes accumulate within the nucleus and that this activity is required in excitotoxic death (O'Hare et al., 2005). Calpain inhibition might preserve the conversion of p35 to p25 and thereby prevent excitotoxic cell death.

Interestingly, a study by Ma et al (2009) reported that statins can selectively modulate calpain cleavage of several target molecules, which are crucial for neuronal survival pathways (Ma et al., 2009) although the mechanism is unknown. Moreover, the PI3K/Akt pathway was identified as one of the crucial upstream signalling sequences activated by the insulin receptor, which is preserved by statins upon NMDA challenge (Ma et al., 2009). Furthermore, it was shown that the PI3K/Akt pathway is also activated by TNF via TNFR2 and that lovastatin mediates neuroprotection via increased the expression of TNFR2 (Dolga et al., 2008; Marchetti et al., 2004).

Furthermore, we could speculate that the modulation of calpain cleavage might be mediated by the NF-κB dependent expression of KCa2 channels, which counteracts Ca^{2+} influx via NMDA receptor. This reduction in intracellular Ca^{2+} levels could be sufficient to suppress calpain activation. In support of this view several studies have shown that the after-hyperpolarization of membranes via K^+ channel openers reduced Ca^{2+} influx and resulted in neuroprotection against Aβ mediated toxicity (Goodman and Mattson, 1996; Mark et al., 1995; Pike et al., 1996). Overall neuroprotective mechanisms and the cellular survival pathways activated by statins are promising drug targets which will can become essential for future therapeutic design.

The strategies discussed so far targeted molecular mechanisms which are involved in a broader range of neurodegenerative disorders and not exclusively AD. A more specific approach aims at one of the major players in AD pathogenesis: the Aβ peptide.

9.5 Modulating Aβ toxicity

According to the "amyloid hypothesis" the Aβ peptide, which accumulates in the brain of patients suffering from AD, is a major pathogenic driving force in this disorder (Selkoe, 2002, 2003). Several strategies have been developed to minimize or reverse the negative effects of Aβ for example reducing Aβ generation, inhibiting of Aβ misfolding and aggregation, or enhancing Aβ clearance (Schenk et al., 1999;

Sigurdsson et al., 2000; Soto, 1999a, b). Approaches aiming at diminishing Aβ levels by inhibition of proteases involved in Aβ generation by APP processing, namely the β- and γ-secretase, have experienced a drawback since these proteases not only generate Aβ but are as well involved in important cellular processes like axonal myelination, synapse maintenance and Notch signalling (Bittner et al., 2009; Lleo et al., 2003; Willem et al., 2006). The challenge here is to develop selective inhibitors which target only the amyloidogenic cleavage without affecting other cellular processes.

Another strategy involves immunization against Aβ-peptides. Aβ vaccination led to amyloid plaque clearance in a mouse model of AD (Schenk et al., 1999). Furthermore, it could be demonstrated that immunization can reverse memory deficits in APP transgenic mice (Dodart et al., 2002). Vaccination against Aβ was the most promising strategy until immunized patients showed strong brain inflammatory responses and a few eventually died (Imbimbo, 2002; Munch and Robinson, 2002).
Potential safety concerns of anti-Aβ vaccines, lead to the development of alternative strategies to reduce the plaque burden in AD. This could be achieved by the inhibition of Aβ aggregation by use of small peptides (also referred as anti-amyloid peptides (AAPs)), which are designed to specifically interact with the protein fragment of Aβ that is undergoing the conformational changes followed by aggregation. AAPs destabilize an undesired conformation of Aβ by adding specific amino acid residues that favor or disfavor the adoption of a particular structural motif (Soto, 1999a, b; Soto et al., 1998). The initially synthesized compounds were peptides of 11 to 5 amino acids targeting the central region of the Aβ peptide and evolved to compounds like LPFFD (iAβ5) and/or LPYFDa.
In **chapter 7 and 8** I evaluate the potential therapeutic properties of short synthetic AAPs, which are designed to neutralize the devastating effect of Aβ peptides. In **Chapter 7** I could demonstrate for the first time that the AAP LPYFDa is a potent inhibitor of Aβ oligomer induced toxicity and is capable to prevent Aβ-induced memory deficits.
Several studies showed that elevated levels of soluble oligomeric Aβ correlate strongly with cognitive decline in AD patients (McLean et al., 1999; Wang et al., 1999). Furthermore, low-n oligomeric assembly of naturally secreted human Aβ alters hippocampal synaptic plasticity by inhibiting long-term potentiation which is associated with impaired memory formation (Walsh et al., 2002). Also the number of dendritic spines was dramatically decreased when neurons were incubated with Aβ oligomers, but not with monomers (Walsh et al., 2002).
Therefore, an important aim of this study was to investigate, whether a compound like LPYFDa is able to prevent Aβ oligomer–induced learning and memory deficits. Indeed a 5-fold molar excess of LPYFDa to Aβ could overcome the detrimental effects of Aβ oligomers on memory. Thus, we provide evidence that so-called β-sheet breaker peptides such as LPYFDa bear therapeutic potential against Aβ-induced memory impairment.

Recently it was demonstrated that intraperitoneally administered LPYFDa is able to cross the blood brain barrier and protects synapses against excitatory action of fibrillar Aβ (Juhasz et al., 2009). A general issue with peptide based drugs for neurological disorders is their rapid degradation *in vivo* by proteolytic enzymes and their poor blood-brain permeability (Adessi and Soto, 2002). These issues were overcome by chemical modifications, like C-terminal amidation and N-terminal carboxylation, which resulted in increased half life *in vivo* and rapid brain up-take (Permanne et al., 2002). Although, our knowledge of the biochemistry in respect to catabolism and brain uptake of certain AAPs has developed greatly in recent years, the detailed mechanisms of action are still poorly understood. Therefore, it is essential to elucidate and study the 3D structure of the Aβ/AAP complex to gain more insight into the molecular dynamics of the Aβ aggregation process.
In **chapter 8** novel AAPs were rationally designed, employing the 3D structure and dynamic behaviour and docking studies of the full length Aβ molecule and the new peptides. The resulting novel peptides were based on the conformational behavior of the $A\beta_{42}$ aggregates previously reported by Masman and co-workers (Masman et al. 2009). The highly hydrophobic sequence Ala30-Ile31-Ile32-Gly33-Leu34, of the $A\beta_{42}$ aggregates was the starting point of the design for the new anti-amyloid peptide PN22. To overcome the high hydrophobicity and improve efficiency some chemical modifications were applied to increase the solubility of the final peptides, such as the introduction of positively charged residues and methyl groups. Furthermore, the N- and C-terminus were blocked by acetylation and amidation respectively which improved cell permeability and an increased stability towards digestion by aminopeptidases. Several studies could show that C-terminal amidation is essential for the biological activity of many neuropeptides and hormones (Fricker, 2005; Kim and Seong, 2001).
Small peptides like LPYFDa and PN22, which are partially homologous to this hydrophobic region, bind with a relatively high affinity to Aβ by similar intermolecular interactions, leading to a competitive replacement of Aβ molecules. The new peptide PN22 and its non methylated version PN20 were synthesized and tested *in vitro* and *in vivo* for the ability of neutralizing Aβ induced toxicity and memory impairment. Similar to LPYFD, PN22 is able to preserve cultured neurons from oligomeric Aβ-induced cell death in a dose dependent manner. Comparable results have been previously reported on PC12 cells using different single N-methyl amino acid containing peptides (Cruz et al., 2004; Hughes et al., 2000). Furthermore, a compound like PN22 is able to prevent Aβ–induced learning and memory deficits. We showed that a 5-fold molar excess of PN22 to Aβ could overcome the detrimental effects of Aβ oligomers on memory. Thus, this study provides evidence that N-methyl containing peptides such as PN22 hold a therapeutic promise against Aβ-induced memory impairment.

The mechanism how AAPs exert their protective effects on cell death and behavior is not yet fully understood. However, there are several options of how AAPs might interact with Aβ. Peptides such as PN22 or LPYFD can directly bind to Aβ, and

thereby prevent possible interactions between Aβ and neuronal membrane proteins and receptors, and in this way neutralize Aβ mediated effects. We could speculate that the AAPs might bind to the monomeric Aβ, thus preventing and/or retarding the formation of toxic oligomers, by disturbing the conformational change that precedes the oligomer formation.
Another possibility would be that the peptides bind to already formed Aβ oligomers and thereby preventing and/or modulating, somehow, their neurotoxic properties. By either of these actions, or both, the neurotoxicity of Aβ is decreased or reverted and the Aβ clearance/degradation processes might be reactivated.
In summary this findings provide evidence on how and where AAPs interact with $A\beta_{42}$ mono- and oligomers that these peptides can neutralizes the neurotoxic effects of soluble Aβ oligomers, and in the present conditions, and as proof of principle, can effectively prevent the oligomer-induced deficits in memory performance. More structural evidence is required to consolidate the proposed mechanism and to further improve the designs. Nevertheless, it is clear that the method of *in silico* design is a valuable tool to develop and predict the efficacy of new drugs like AAPs for treatment of AD.

9.6 Concluding remarks

Therapies directed against specific aspects of AD pathogenesis like neuroinflammation, excitotoxicity and Aβ induced damage are all considered to alleviate problems associated with the disease. In this thesis several compounds were evaluated (Statins, Calpain Inhibitors, Anti-Amyloid Peptides) targeting different molecular mechanisms underlying AD. In conclusion it could be demonstrated that all tested compounds bear potential to intervene in certain aspects of AD pathology. However, at this stage it remains elusive, which type of treatment might be the best approach to halt or slow the progression of the disease, since our results have been obtained from cell culture and animal models for AD (van Dam et al., 2006) and have not been tested in human patients. Moreover, it is questionable whether a single drug in particular, will be able to stop or prevent such a complex multifactorial disorder like Alzheimer's disease.
From that point of view one might speculate that for an effective treatment it might be necessary to target multiple molecular processes underlying the AD pathology. Depending on the stage of the disease combined therapeutic approaches using AAPs with statins or calpain inhibitors could be worthwhile investigating. The AAPs would intervene early in the cascade of pathogenic events by neutralizing harmful actions of Aβ, whereas the calpain inhibitors interact downstream from Aβ. Since both drugs target different molecular mechanisms, their actions might be complementary. Lovastatin itself has already pleiotropic actions affecting several molecular pathways, which are involved in the progression of AD. A useful supplement for a neuroprotective lovastatin treatment would be a selective activator

of TNFR2 and/or a selective inhibitor of TNFR1. Finally, one should not disregard the importance of early diagnostic tools (e.g. CSF biomarkers (Le Bastard et al., 2009)) for the detection of AD long time before the first symptoms occur in order to enable effective treatment.

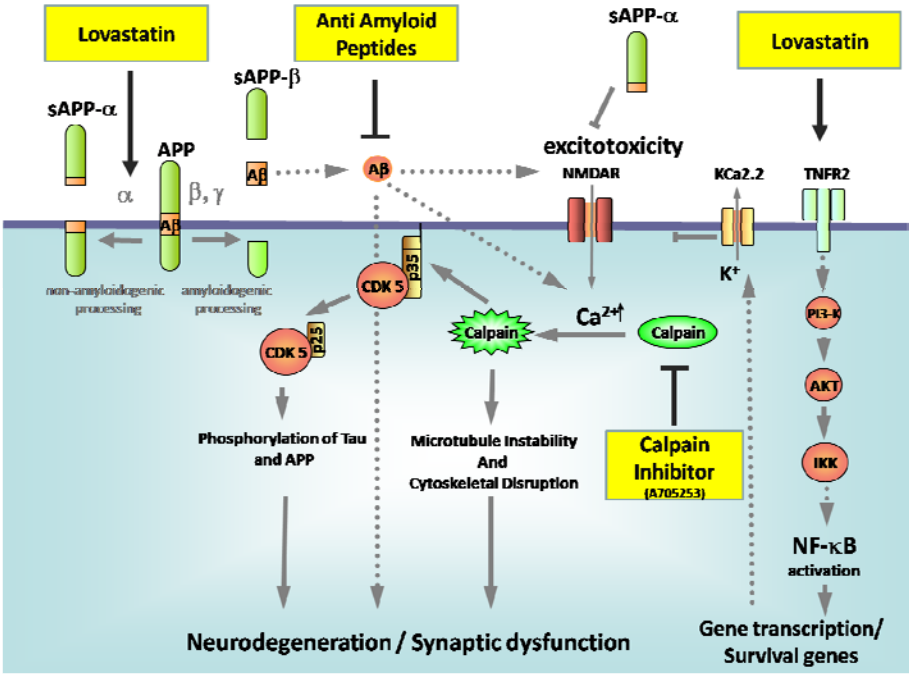


Figure 9.1. *Schematic overview of the neuroprotective strategies for Alzheimer' s disease. Lovastatin treatment leads to an increased expression of TNFR2. Activation of TNFR2, can directly, lead to sustained activation of NF-κB to initiate transcription of NF-κB dependent anti apoptotic genes such as KCa2.2. Furthermore, Lovastatin triggers the non amyloidogenic APP cleavage via increased α-secretase activity. The resulting sAPPa has excitoprotective properties. Calpain inhibitor A705253 prevents cleavage of substrates which are essential for normal cellular functioning. Anti Amyloid Peptides bind to soluble monomeric or oligomeric Aβ and neutralize its harmful properties.*

9.7 Future Perspectives

A key challenge in current AD research has become to understand precisely what happens at the earliest stages of disease. In particular it is of great importance to identify and investigate factors, which are responsible for the onset of sporadic AD as this form accounts for majority of AD cases (Bouwman et al., 2009; Galimberti et al., 2003; Galimberti et al., 2006; Henneman et al., 2009).

Since many neurodegenerative disorders including AD mainly affect elderly people it seems plausible that changes, which occur during the ageing process, make the brain more susceptible to diseases. Cytokine profile, inflammation response and the expression of several proteins are altered during the ageing process and many of these proteins are crucial for maintaining normal physiological functioning (Toescu et al., 2009; von Bernhardi er al., 2010; Liu et al., 2010, Xu et al., 2010).
Significant reduction of particular proteins during ageing can have devastating consequences. For instance the protein levels of the Aβ degrading enzyme neprilysin are drastically decreased during ageing, which reduces Aβ clearance and promotes its accumulation. In turn elevated Aβ levels can lead to more activated microglia, which release proinflammatory cytokines such as TNF. In a vicious cycle, cytokines can trigger the amyloidogenic APP processing by promoting BACE1 expression, which leads to more Aβ. As a consequence of elevated Aβ levels, oxidative stress, and inflammation result in disturbed calcium homeostasis. In combination with age related decrease of calcium binding "buffer" proteins, such as calbindin, calretinin and parvalbum, neuronal cells are more susceptible for cytotoxic insults (Pike et al., 1995; Harkany et al., 1995; Yatin et al., 1999).

Population based studies on ageing such as the Rotterdam study suggest that lifestyle can also influence the risk for developing AD. Moderate alcohol consumption has been reported to rather decrease the risk (Ruitenberg et al., 2002) contrary to smoking which seems to increase the risk for developing AD (Reitz et al., 2007). Another important factor for attenuating the risk of AD may be physical exercise. Exercise increases brain derived neurotrophic factor, promotes sprouting of dendrites and generation of new synapses (Vaynman et al., 2003; Ploughman et al., 2007; Fischer et al., 2007).
Further characterization of the ageing process and specific cellular signalling pathways may provide us with novel drug targets and potential pharmacological interventions to combat AD and enable a healthy ageing.

Nederlandse Samenvatting

De ziekte van Alzheimer is de meest voorkomende vorm van dementie. In Nederland lijden ongeveer 130.000 mensen aan deze progressieve hersenziekte.
Sinds de beschrijving van de ziekte 100 jaar geleden door Alois Alzheimer, is de oorzaak van de ziekte nog altijd niet precies bekend.
Tot nu toe zijn o.a. veroudering, oxidatieve stress, verstoorde ionenhuishouding, verminderde hersendoorbloeding, neuronale ontstekingen en genetische afwijkingen geïdentificeerd als belangrijkste factoren die bijdragen aan de pathogenese van de ziekte.
De karakteristieke pathologie van Alzheimer bestaat uit neuronale ontsteking, afsterven van zenuwcellen en ophoping en verklontering van het amyloide-β eiwit. Dit leidt uiteindelijk tot het fysieke verval van de hersenen welke gepaard gaat met klinische symptomen zoals algemene cognitieve achteruitgang, geheugenverlies en veranderingen in de persoonlijkheid.

Het doel van dit proefschrift is enkele nieuwe therapeutische strategieën te ontwikkelen en te evalueren gebaseerd op de huidige kennis van de ziekte van Alzheimer. Hierbij probeer ik de verschillende ziekteverwekkende moleculaire processen af te remmen of te vertragen.

Epidemiologische studies naar cholesterolverlagende middelen suggereren dat deze medicijnen ook bruikbaar zijn bij de preventie of vertraging van Alzheimer. Lovastatine is één van deze middelen welke, naast de cholesterolverlagende eigenschappen, een stimulering van de receptor 2 van de ontstekingsfactor Tumor Necrose Factor (TNFR2) oproept. Uit onderzoek in cel culturen (*in vitro*) is gebleken dat deze receptor een beschermende werking heeft op celdood veroorzaakt door overstimulatie van zenuwcellen met glutamaat, een proces dat aangeduid wordt met "excitotoxiciteit". Op moleculair nivo is verder aangetoond dat de signaaloverdracht van TNFR2 leidt tot activatie van de transcriptie factor NF-κB (Nuclear Factor kappa B) via Protein Kinase B (PKB/Akt) waardoor een aantal neuroprotectieve genen tot expressie komen. Hierdoor zijn verschillende schakels binnen deze route van signaaloverdracht een doelwit voor potentiële medicijnen tegen neurodegeneratieve aandoeningen. De neuroprotectieve werking van lovastatine via TNFR2 is tot nu toe alleen aangetoond op zenuwcellen in culture (*in vitro*) maar nog niet in levende organismen (*in vivo*).
In **hoofdstuk 2 is** de beschermende werking van lovastatine bestudeerd in een *in vivo* model voor neurodegeneratie waarin de cholinerge neuronen in de nucleus basalis magnocellularis (NBM) in muizen uitgeschakeld is door toxische NMDA injecties. De dysfunctie en het verlies van de cholinerge neuronen en hun corticale projecties behoren tot een van de eerste pathologische manifestaties bij het ontstaan van Alzheimer wat resulteert in geheugenstoornissen. In dit onderzoek heb ik aangetoond dat voorbehandeling met lovastatine de degeneratie van cholinerge vezels kan voorkomen en daarmee de cognitieve achteruitgang remt.

Daarnaast heb ik *in vivo* bevestigd dat lovastatine-gemedieerde neuroprotectie afhankelijk is van het PKB/Akt signaalpad doordat de inhibitie van de PKB/Akt met LY294002 de neuroprotectieve werking blokkeert. Uit deze studie concludeer ik dat behandeling met lovastatine bescherming biedt tegen neuronale schade onder excitotoxische omstandigheden zoals in veel neurodegeneratieve ziekten voorkomt.

De transcriptiefactor NF-κB reguleert de expressie van (small conductance) calcium-geactiveerde kalium (KCa2) kanalen. Deze KCa2 kanalen verminderen de prikkelbaarheid van de zenuwcel wat leidt tot neuroprotectie tegen neuronale overstimulatie (excitotoxiciteit). In het algemeen wordt excitotoxiciteit beschouwd als een belangrijk mechanisme betrokken bij neurodegeneratie van het centrale zenuwstelsel zoals het geval is bij een hersenbloeding, trauma, Alzheimer en andere neurologische aandoeningen.
In **hoofdstuk 3** heb ik onderzocht of TNF-α-gemedieerde neuroprotectieve signalering, veranderingen induceert in de expressie van KCa2 kanalen. Ik vond een verhoogde expressie van KCa2 kanalen type 2 (KCa2.2) na behandeling met TNF in een tijdsafhankelijke manier. Deze toename blijkt afhankelijk van TNFR2 en NF-κB activering. De expressie van KCa2.1 en KCa2.3 kanalen blijft onveranderd na behandeling met TNF.

Op fuctioneel niveau leidt de specifieke activering van KCa2.2 door NS309 of CyPPA tot neuroprotectie tegen glutamaat geïnduceerde overstimulatie. In tegenstelling tot de activering leidt de blokkade van KCa2.2, met apamin of de uitschakeling van het KCa2.2 kanaal met siRNA, tot vermindering van het neuroprotectieve effect. Uit deze studie concludeer ik dat behandeling van primaire corticale neuronen met TNF-α leidt tot een verhoogde KCa2.2 kanaal expressie in neuronen wat ze beter bestand maakt tegen excitotoxische celdood.

Calpaine, een calcium-afhankelijke cysteïne protease, is onderdeel van het NMDA receptor-geïnduceerde excitotoxisch signaalpad. Ook het Aβ eiwit is schadelijk voor neuronen en deze toxiciteit is -althans gedeeltelijk - gemedieerd via de NMDA receptor. De **hoofdstukken 4 en 5** van dit proefschrift zijn gericht op de vraag of remming van Calpaine neuronale celdood kan voorkomen. Hiervoor heb ik in een ziekte-relevant diermodel, gebaseerd op excitotoxische laesies van de cholinerge nucleus basalis magnocellularis middels NMDA of Aβ, onderzocht of de bijbehorende geheugenstoornissen te voorkomen zijn door behandeling met een Calpaine remmer. Excitotoxische laesies van de nucleus basalis leiden tot verminderde prestaties in leer- en geheugentaken. Behandeling met de Calpaine remmer A-705253, voorkomt deze geheugenafwijking in een dosisafhankelijke manier. Analyse van de cholinerge vesels in de corticale schors van de dieren met leasie bevestigen deze resultaten. Daarnaast blijkt uit immunohistochemische analyse van geactiveerde microglia cellen dat de remming van Calpaine ook tot een verminderde ontstekings reaktie leidt die meestal samen gaat met neurodegeneratieve ziektes.

Uit dit onderzoek kan geconcludeerd worden dat de remming van Calpaine een waardevolle strategie is in de preventie van oligomeer Aβ en/of excitotoxiciteit-geïnduceerde neuronale celdood. Op deze manier wordt ook de hiermee samenhangende cognitieve achteruitgang voorkomen zonder fysiologische neuronale functies te verstoren. De remming van Calpaine activiteit is een nieuwe en veelbelovende benadering in de behandeling van diverse neurodegeneratieve aandoeningen.

Ontstekingsreacties zijn een kenmerk van veel, zo niet alle chronische ziekten, waaronder de ziekte van Alzheimer en diabetes mellitus.
Een aantal recente studies laten zien dat type twee diabetes het risico op het ontwikkelen de ziekte van Alzheimer verhoogt. Deze kennis heeft geleid tot onderzoek naar de gemeenschappelijke processen die een relatie tussen deze twee ziektes kan verklaren. In **hoofdstuk 6** geef ik een overzicht van de literatuur die betrekking heeft op de rol van ontsteksreacties in zowel Diabetes mellitus als de ziekte van Alzheimer en de mogelijke functie van de transcriptie factor NF-κB.

Een verkeerde vouwing, oligomerisatie en ophoping van het amyloïd-beta (Aβ) peptide wordt algemeen beschouwd als een centrale gebeurtenis in de pathogenese van de ziekte van Alzheimer. Recente studies hebben oplosbare Aβ oligomeren geïdentificeerd als de belangrijkste ziekteverwekkers. Deze bevindingen zijn bevestigd door studies die laten zien dat deze neurotoxische Aβ aggregaten de synaptische plasticiteit verstoren en de lange termijn potentiatie (LTP) remmen. Een veelbelovende therapeutische strategie in de strijd tegen AD is het toedienen van kleine synthetische peptiden die zodanig zijn ontworpen dat ze aan specifieke regio's van Aβ oligomeer binden en de verwoestende eigenschappen van oligomere Aβ neutraliseren. In **hoofdstuk 7** onderzoek ik de eigenschappen van het pentapeptide LPYFDa op Aβ-geïnduceerde geheugenstoornis en celdood. *In vitro* heb ik aangetoond dat neuronen die behandeld zijn met LPYFDa beschermd zijn tegen Aβ-geïnduceerde celdood. Bovendien, kan LPYFDa een Aβ geinduceerde geheugenstoornis *in vivo* voorkomen. Dit is de eerste studie die aantoont dat een anti-amyloid peptide als LPYFDa geheugenfuncties kan beschermen tegen door Aβ oligomeer veroorzaakte geheugenuitval.

Als vervolg op de studie in hoofdstuk 7 is in **hoofdstuk 8** met behulp van een driedimensionaal computermodel het Aβ aggregatie proces bestudeerd om meer inzicht te krijgen in de moleculaire interacties tussen de aggregatie remmende peptide en het Aβ oligomeer complex. Als resultaat van deze computersimulaties is een nieuw peptide ontworpen (PN22), gesynthetiseerd en *in vitro* en *in vivo* geëvalueerd op Aβ neutraliserende eigenschappen. *In vitro* heb ik aangetoond dat neuronen behandeld met PN22, beschermd zijn tegen Aβ-veroorzaakte celdood. Bovendien, kan PN22 (net als LPYFDa) Aβ geïnduceerde geheugenstoornis *in vivo* voorkomen.

Therapieën die gericht zijn op specifieke aspecten van de pathogenese van Alzheimer, zoals neuronale overstimulatie en schade veroorzaakt door Aβ, zouden de problemen die geassocieerd worden met de ziekte kunnen verlichten en bestrijden. In dit proefschrift worden verschillende middelen geëvalueerd, (waaronder statines, calpaine remmers en anti-amyloïde peptiden), welke gericht zijn op de verschillende onderliggende moleculaire mechanismen van de ziekte van Alzheimer.
Echter, in dit stadium is het nog onduidelijk welk type behandeling de beste aanpak is om de progressie van de ziekte te vertragen of te stoppen. De resultaten uit dit onderzoek zijn verkregen uit celkweek en diermodellen voor Alzheimer en nog niet getest in patiënten. Bovendien blijft het de vraag of dergelijke geneesmiddelen in staat zullen zijn om alle aspecten van deze complexe ziekte te voorkomen of te stoppen.
Vanuit dit oogpunt kan men speculeren dat voor een effectieve behandeling het noodzakelijk is om op meerdere moleculaire processen in te grijpen. Ten slotte moet men niet voorbijgaan aan het belang van goede diagnostische instumenten die de ziekte vroegtijdig opsporen voordat de eerste symptomen zich voordoen om een doeltreffende behandeling mogelijk te maken.

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Curriculum Vitae

Ivica Granic was born 16th of May 1979, as son of Croatian immigrants in Marbach (am Neckar), Germany. He finished lower secondary school in 1994 followed by two years of vocational school in Ludwigsburg (Germany). In 1996 he continued his education by visiting the technical high school for natural sciences (Kerschensteinerschule) in Stuttgart (Germany), which included a training program for chemical-technical assistants. After successful graduation in 1999, Ivica studied technical biology at the University of Stuttgart, with cell biology and animal physiology as major subjects. During his study he worked part-time as a student assistant at the institute of cell biology and immunology at the same University. In his bachelor phase he became very interested in neurobiology and in particular biological processes underlying neurodegenerative disorders. For his student research project Ivica left Germany to do research on the distribution of tumor necrosis factor and its receptors in Alzheimer's disease under the supervision of Dr. Ulrich Eisel in Groningen (the Netherlands) and Prof. Klaus Pfizenmaier in Stuttgart. During his master project he investigated beta-sheet breaking peptides as potential therapeutics for Alzheimer's disease in Stuttgart under the supervision of Dr. Ulrich Eisel (Groningen) and Prof. Peter Scheurich in Stuttgart.

After his graduation in 2005 Ivica started a 4 years PhD project in the Group of Prof. Paul Luiten at the Department of Molecular Neurobiology in Groningen, on neurodegenerative mechanisms and therapeutic strategies in Alzheimer's disease. The completion of this project led to the present dissertation titled 'Neurodegenerative mechanisms in Alzheimer's disease – amyloid aggregation, neuroinflammation and apoptosis'.

Since 1st of January 2010 he works as a post doc fellow in the group of Prof. Gerard Martens at the department of Molecular Animal Physiology, part of the Donders Center for Neuroscience and the Nijmegen Center for Molecular Life Sciences (NCMLS) at the Radboud University in Nijmegen (Netherlands). In his current project he investigates the role of different BDNF splice variants on synaptic plasticity and cell survival.

List of publications

Full Papers

Ivica Granic, Csaba Nyakas, Paul G.M. Luiten, Ulrich L.M. Eisel, Laszlo G. Halmy, Gerhard Gross, Hans Schoemaker, Achim Möller, Volker Nimmrich. Calpain inhibition prevents amyloid-beta-induced neurodegeneration and associated behavioral dysfunction in rats. Neuropharmacology (submitted).

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Volker Nimmrich, Robert Szabo, Csaba Nyakas, Ivica Granic, Klaus G.Reymann, Ulrich H. Schroder, Gerhard Gross, Hans Schoemaker, Karsten Wicke, Achim Moller, Paul Luiten. Inhibition of Calpain Prevents N-Methyl-D-aspartate-Induced Degeneration of the Nucleus Basalis and Associated Behavioral Dysfunction. J Pharmacol Exp Ther 2008 327: 343-352.

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